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Lipopolysaccharide-induced signal transduction in macrophages: Mechanisms of cellular responsiveness

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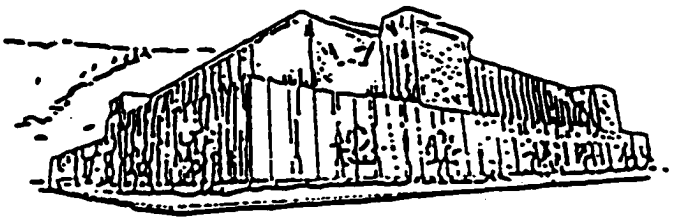

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Lipopolysaccharide-induced signal transduction in macrophages: Mechanisms of cellular responsiveness

By

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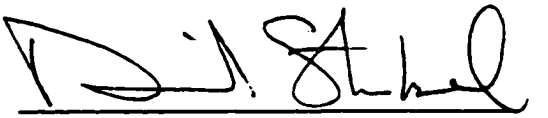

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Lipopolysaccharide-Induced Signal Transduction in Macrophages: Mechanisms of Cellular Responsiveness. (131 pp.)

Committee Chair: George L. Card, Ph.D.

Signal transduction pathways in macrophages activated by lipopolysaccharide (LPS) are only beginning to be elucidated. The first step involves recognition of LPS by the cells primarily by the LPS receptor, CD14. Different LPS and lipid A preparations, as well as lipoteichoic acids from Gram positive organisms, bind to CD14 and activate signaling. However, they showed a wide range of potencies in activating arachidonic acid metabolism. These data combined with current theories on supramolecular conformations of bacterial amphiphiles, suggest a model of potency based on aggregate conformation and the availability of monomers for binding to CD14. Although not conclusive, this model provides a basis for exploration of variations in responsiveness to bacterial amphiphiles.

Ceramide is an important second messenger in immune cells, and it appears to share signaling components with LPS pathways. Studies on the hypothesis that LPS acts by mimicking ceramide have provided many clues to mechanisms of intracellular modulation of responsiveness. LPS and ceramide both activated arachidonic acid metabolism, but only LPS induced the production of prostaglandin E_2. This suggests a divergence in signaling pathways that may be related to pool compartmentalization of the signaling enzymes or their substrates. LPS, but not ceramide, was able to desensitize cells to subsequent challenge. This difference could either be due to a lack of a shared regulatory mechanism, or to an autocrine/paracrine effect related to the activation of prostaglandin production.

Responsiveness is also determined by the phosphorylation state of tyrosine kinases of the src family. CD45 protein tyrosine phosphatase was shown to be involved in LPS-induced signaling through the use of anti-CD45 monoclonal antibodies. Although the antibodies and their isotype controls had little or no effect on their own, anti-CD45 significantly increased (2-fold) the release of arachidonic acid metabolites, including prostaglandin E_2 following LPS challenge. This effect was shown to be primarily involved in signaling through CD14, rather than CD14-independent signaling.

ACKNOWLEDGEMENTS

In the laboratory of Dr. George Card I found a place to learn to do good science, to deepen my love and enthusiasm for science in general and cell physiology in particular, and to develop the confidence necessary to pursue a future in a complex and challenging field. More importantly, I found a place to heal and grow. For these things, he has my deepest appreciation and my highest esteem. I also extend sincere thanks to my other committee members, Mike Minnick, Walt Hill, Jim Gannon, Ed Walker, and Stuart Hall, who always made their expertise and advice available, and helped me find a way past the obstacles. I had excellent technical support from Ribi ImmunoChem Research in Hamilton, especially from Linda Griggs and Kevin Floyd. Several fellow graduate students took extra time to help me in so many ways: but I especially thank Angelika Longacre, Bruce Wielinga, and Steve McAllister for their friendship and for all of the computer lessons. To my parents, I send special thanks for their support and encouragement. Finally, I dedicate this work to my son, Justin, for his honesty, his patience, his love, and his wonderful sense of humor that can always put things in perspective.

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AA	Arachidonic acid, a C_{20} polyunsaturated fatty acid.
CAPK	Ceramide Activated Protein Kinase
CAPP	Ceramide Activated Protein Phosphatase
COX	Cyclooxygenase, the initial enzyme in prostaglandin synthesis.
D3	1α, 25 – dihydroxyvitamin D3, a maturation agent for THP-1 cells.
DAG	Diacylglercerol
DEX	Dexamethasone, PLA_2 inhibitor.
DPL	Diphosphoryl Lipid A
ECL	Enhanced Chemiluminescence, a sensitive method to develop a Western blot photographically.
EIA	Enzyme ImmunoAssay
ELISA	Enzyme Linked Immunosorbent Assay
FALS	Forward Angle Light Scatter
FBS	Fetal bovine serum
FcR	A receptor which binds the Fc portion of antibodies.
FITC	Fluorescein isothiocyanate, a fluorescent tag for antibodies.
IL	Interleukin, a family of cytokines from leukocytes.
KDO	Ketodeoxyoctonate, part of the inner core of LPS.
LBP	Lipopolysaccharide binding protein
LTC_4	Leukotriene C_4, a peptidyl-leukotriene.
mAb	Monoclonal antibody
MAPK	Mitogen-Activated Protein Kinase
MLA	Monophosphoryl Lipid A, original name.
MPL	Monophosphoryl Lipid A, solubilized in TEA
MPL-S	Monophosphoryl Lipid A, solubilized in EtOH
$NaVO_4$	Sodium Vanadate, a tyrosine phosphatase inhibitor
NFkB	Nuclear Factor, kappa B.
PA	Phosphatidic Acid
PC	Phosphatidylcholine
PEC	Peritoneal exudate cells, usually refers to harvested macrophages
PGE_2	Prostaglandin E_2
PhAsO	Phenylarsine oxide, a protein tyrosine phosphatase inhibitor
PI	Phosphatidylinositol
PL	Phospholipid
PLA_2	Phospholipase A_2
PMA	Phorbol Myristate Acetate, a DAG analog.
PMN	Polymorphonuclear Phagocyte, neutrophil.
PTK	Protein Tyrosine Kinase
PTPase	Protein Tyrosine Phosphatase
SM	Sphingomyelin
TEA	Triethylamine
TNF	Tumor Necrosis Factor

CHAPTER 1

OVERVIEW OF MACROPHAGE ACTIVATION AND DESENSITIZATION

The role of the macrophage in the vertebrate immune system.

The vertebrate immune system functions as a dynamic balance between states of activation and states of tolerance, and many components of the system embody mechanisms to respond to various signals in a discriminating manner. Errors in discrimination or deviations in the delicate balance can lead to responses that are damaging to the organism. If poorly responsive, the immune system is ineffective in its protective role, but if overly responsive it can induce pathological consequences. Macrophages have proven to be at the hub of this process, playing significant roles in both cellular and humoral immune responses. They are large, adherent, mononuclear phagocytic cells which differentiate from circulating monocytes and migrate into various tissues. From this position of influence, the macrophage's activities must be carefully regulated in order to maintain a balance of responsiveness. Depending on the stimulus, several different signaling pathways lead to a variety of responses, including cytokine release (especially TNFα, IL_1, IL_6, IL_8), oxidative burst, increased phagocytosis, and arachidonic acid metabolism. Macrophage activation is being studied regarding its involvement in multiple pathological responses including septic shock, asthma, stroke, and atherosclerosis (5,13,71,81). It is therefore conceivable that treatments or prevention of these diseases may evolve through the

exploration of mechanisms that influence the balance between the beneficial and pathological sequelae of macrophage activation.

Overview of signal transduction pathways

In order to understand the mechanisms of macrophage activation, it is necessary to understand the basics of signal transduction, which entails the ability of a cell to detect and respond to extracellular signals. Signal transduction through cell surface receptors can be categorized into three large general groups. In one well characterized pathway, a transmembrane receptor activates a G (GTP-binding) protein, which then alters the activity of adenylate or guanylate cyclase. Activation of these enzymes results in the generation of second messengers, including cyclic adenosine monophosphate (cAMP) or cyclic quanosine monophosphate (cGMP). In another family of pathways, the receptor activates a specific phospholipase C, which cleaves a phosphodiester-linked side group from a phospholipid or sphingolipid. The cleavage products, such as inositol triphosphate, diacylglycerol, or ceramide can then serve as second messengers affecting a cellular response. Finally, some receptors activate protein kinases and phosphatases which in turn can initiate the cellular response by altering phosphorylation of target proteins which are often enzymes or regulatory elements. This is the primary signaling system used by the antigen-binding receptors of lymphocytes. Each of these systems has mechanisms for shutting down the response when the signal is no longer present or a second signal overrides the first. Usually this is not a simple on/off switch, but rather a change in the balance or ratio between opposing signals that affects the final outcome. Therefore, the activation

state of a cell depends on the expression of receptors, availability of second messengers, and the ability to shut down the response.

Definition of activation states

Studies have shown that macrophages respond very differently depending on anatomical location, the nature of the stimulus, and the cell's state of differentiation. Strictly speaking, macrophage activation states probably lie on a continuum encompassing several hundred "forms of activation" (92). Attempts to characterize them as macrophage subsets according to location or surface markers have been unsuccessful (36). For the purpose of this dissertation, only three states will be defined. These are "responsive", "activated", and "desensitized".

A responsive macrophage is competent to respond to signals. It might be considered to be in a state of vigilance, appropriate for the macrophage's critical role in detection. These cells respond to a stimulus by releasing high levels of cytotoxic and biologically active substances, many of which serve to signal other cells. Stimuli can include bacterial cell wall components, cytokines, and particles that are phagocytized. The activated macrophage can be further stimulated with synergistic signals and induced to release higher levels of substances such as tumor necrosis factor α (TNFα) and interleukins, but the mechanisms of this additive response are not clearly understood (1,17,39). However, it is the excessive or unbalanced production of these autocrine and paracrine mediators which has been implicated in multiple disease states.

The desensitization phenomenon

At the other end of the continuum is a macrophage that has been desensitized and is hyporesponsive. There are several mechanisms that may induce this state. A fairly specific hyporesponsiveness is induced simply by saturating concentrations of ligand or the internalization of receptors, making them unavailable for binding (17). This requires a very specific receptor-ligand relationship, and the cell remains responsive to other stimuli. Similarly, a means to down-regulate responsiveness to a specific stimulus is to shed the receptor, and decrease its surface density (9). A more generalized hyporesponsiveness is induced in macrophages by glucocorticoids (110). The pathway is unclear, but it appears to be a natural mechanism for shutting down a response by decreasing the activity of enzymes such as the phospholipases. There is some evidence that this involves phosphorylation or dephosphorylation (47), and/or the release of autocrine mediators (110). A third mechanism of desensitization has been demonstrated as a fairly specific hyporesponsiveness by macrophages to a second exposure to bacterial cell wall amphiphiles such as lipopolysaccharide (LPS) from Gram negative microorganisms (42,96), and lipoteichoic acid (LTA) from Gram positive microorganisms (16). The state can be induced by concentrations of LPS far below saturation (17,28), and the cells remain responsive to other stimulators such as phorbol myristate acetate (PMA) which bypass early steps in the signaling cascade (16). This suggests that the mechanism of desensitization is an early event that lies between receptor binding and distal signaling. Others have provided some evidence that the process requires the release of autocrine mediators (28,41,76,84), although the

data are not consistent enough to determine the precise factor(s) involved. Desensitization is characterized by dramatic reductions in the amount of products released after subsequent challenge to, or below, constitutive levels.

Signal transduction in the activation of macrophages by LPS

Bacterial lipopolysaccharide (LPS) is a potent activator of macrophages, but the signal transduction pathways for its numerous effects have not been clearly defined. LPS activates multiple pathways, resulting in the release of cytokines, eicosanoids, and oxygen free radicals. Macrophages have several surface binding proteins for LPS, but only a few appear to be involved in signal transduction. In fact, they appear to be able to respond to LPS via both receptor-dependent and receptor-independent pathways (68,84,106,108). Differential activation of these pathways may depend on the concentration of LPS, its supramolecular structure as it is presented to the cells, and/or the presence of LPS binding proteins. Lipid A derivatives have been designed that retain immunostimulatory properties, but lack endotoxicity. These remain able to desensitize cells to LPS challenge, and are being explored as preventative treatments for pathological inflammatory reactions.

The primary receptor for LPS actually appears to bind a complex consisting of LPS and LPS binding protein (LBP). LBP is an acute phase protein found in the serum, that binds LPS in an apparent 1:1 stoichiometry. This offers a unique way to present LPS to cells. As an amphiphilic lipid, LPS is normally found in aggregates such as micelles. The receptor, CD14, is a glycosylphosphatidylinositol-linked glycoprotein that has been shown to play a role in LPS-mediated macrophage

activation (91,98) and desensitization (57,70). Since it lacks a cytosolic tail, it is still unclear how it participates in signal transduction. However, CD14 is among several GPI-anchored proteins that are capable of mediating cell activation when cross-linked with monoclonal antibodies (67,88). CD14 has also been shown to co-precipitate with associated proteins (91). Deacylated LPS binds CD14, but does not elicit responses, suggesting that another molecule associated with CD14 is required to transduce the signal (57). In fact, Vasselon, et al. have recently shown that trypsin treatment reduced responsiveness to LPS in PMNs without any change in cell surface expression of CD14 (95). It is possible, therefore, that there is a receptor complex on macrophages which is analogous to the complexes on T cells and B cells. Signaling following binding to CD14 has been shown to include protein tyrosine phosphorylation (43,60,98), release of cytokines including TNFα, IL-1, and IL-6 (57,98), calcium fluxes and release of oxygen free radicals (66). Involvement of CD14 in the release of eicosanoids is inferred by the dramatic increase in LPS-induced release of labeled arachidonic acid from THP-1 cells following induction of CD14 expression (Fig. 7 in Chapter 2).

Arachidonic acid metabolism

Arachidonic acid, a C20 polyunsaturated fatty acid ($C_{20:4}$) is commonly esterified at the *sn*-2 position of membrane glycerophospholipids. When released, usually by phospholipase A_2 (PLA_2), arachidonic acid becomes a substrate for several metabolic enzymes. Arachidonic acid and its family of metabolites constitute a very important group of paracrine and autocrine mediators called eicosanoids. Eicosanoids are

rapidly released from macrophages following activation with various agents including LPS and PMA. Two of the major eicosanoids released from macrophages are prostaglandins (cyclo-oxygenase pathway) and peptidyl-leukotrienes (lipoxygenase pathway) (31,33,48,105). The ratio of these two lipid families appears to be important in the regulation of the immune response (100,105). Therefore, the study of arachidonic acid metabolism in macrophages is critical to the understanding of regulatory mechanisms in the cellular immune response.

RESEARCH OBJECTIVES

This thesis focuses on the signal transduction leading to arachidonic acid metabolism in THP-1 monocytic cells, with an emphasis on regulatory mechanisms such as the desensitization phenomenon. Treatment strategies for inflammatory responses associated with Gram negative bacterial infections have ranged from palliative therapies, to cytokines given as pharmaceuticals, to monoclonal antibodies generated against LPS. Despite promising results in *in vitro* studies and animal models, none of these strategies has had much success in clinical studies to date. It seems valid to explore regulatory systems already in place in the immune system as possible mechanisms to exploit as therapeutic strategies. However, such studies will require cell systems that provide good models for human macrophage activation, a better understanding of how cells respond to LPS including specific signal transduction pathways, and elucidation of those pathways' regulatory mechanisms.

Major Objective #1: Characterization of the human monocytic cell line, THP-1, as an appropriate cell system for exploring macrophage activation, especially arachidonic acid metabolism.

Specific Aims:

1. To study basic growth characteristics and determine ideal growth conditions.

2. To observe viability and morphological changes following treatments using trypan blue exclusion and microscopy to determine that cell losses due to death or adherence would not significantly impact assay results.

3. To measure lipid composition and pool labeling by thin layer chromatography and liquid scintillation counting to demonstrate that treatments did not cause alterations in these parameters that would impact assay results.

4. To measure surface expression of CD14 using flow cytometry to determine whether treatments affected expression of this LPS receptor.

Major Objective #2. Compare the activation and desensitization of macrophages by various bacterial amphiphiles, using the hypothesis that the supra-molecular structure of LPS and related molecules likely has an important influence on potency.

Specific Aims:

1. To measure ^{3}H-arachidonic acid release from macrophages treated with various forms of LPS, lipid A, and LTA, the bacterial amphiphiles involved in septic shock.

2. To demonstrate desensitization of macrophages by these amphiphiles for arachidonic acid metabolism and release of pro-inflammatory cytokines.

3. To compare PGE_2/LTC_4 ratios released from treated cells, using EIA, to determine whether patterns can be seen in terms of the ability to desensitize cells to subsequent challenge.

Major Objective #3. Compare the effects of LPS and ceramide as activators of arachidonic acid metabolism, based on the current thinking that LPS may mimic

ceramide as a second messenger. This model, if valid, would define many of the signal transduction components in LPS pathways that have otherwise proven elusive. It could also be very important in the understanding of septic shock syndrome due to the induction of apoptosis by ceramide and the recent evidence that a major part of septic shock is widespread endothelial apoptosis of the microvasculature of multiple organs. Development of the LPS/ceramide mimicry model has been based primarily on studies of cytokine release, and similarities between signaling by TNF and LPS. It was therefore important to explore the model in terms of arachidonic acid release.

Specific Aims:

1. To determine whether exogenous cell-permeable ceramides and/or sphinomyelinase would activate arachidonic acid metabolism, and to compare that with LPS activation.

2. To measure PGE_2/LTC_4 ratios released following these treatments to compare activation of these pathways (cyclo-oxygenase and 5'-lipoxygenase).

3. To determine whether cell-permeable ceramide or sphingomyelinase could desensitize cells to subsequent challenge.

4. To correlate eicosanoid ratios with the ability to desensitize.

Major Objective #4. Determine whether or not the CD45 protein tyrosine phosphatase plays a role in the activation of macrophages by LPS. The hypothesis was that CD14 might be part of a receptor complex on macrophages similar to antigen receptor complexes on T cells. CD45-deficient T cells are hyporesponsive to antigen binding due to hyperphosphorylation of the tyrosine kinases involved in the signaling pathway. It seemed possible that phosphorylation- dephosphorylation dynamics might

therefore play a role in the desensitization phenomenon in macrophages by a similar mechanism.

Specific Aims:

1. To show expression of CD45 on THP-1 cells using flow cytometry, and to determine whether LPS altered this expression either in terms of percent of cells staining positive, or mean fluorescence.

2. To use specific monoclonal antibodies (mAb) to CD45 to determine their effect, if any, on arachidonic acid metabolism and cytokine release induced by LPS, either by binding or by being cross-linked.

3. To correlate CD45 involvement with signaling through the CD14 pathways by performing assays under conditions favoring this pathway, and by using an anti-CD14 mAb which binds the LPS binding site and activates the LPS pathways.

4. To determine whether CD45 plays a role in the desensitization phenomenon by adding anti-CD45 mAb during the desensitization period of the assays.

5. To use Western blotting to detect changes in the phosphorylation state of CD45 on cells following treatment with LPS, since autodephosphorylation is an indicator of enzyme activity for CD45.

The experimental studies addressing these objectives are presented in the following chapters.

CHAPTER 2

Characterization of THP-1 Cells as a Human Macrophage Model for Activation of Arachidonic Acid Metabolism

Introduction

Developing a model for studying macrophage activation is challenging due to the heterogeneity of this cell type and the difficulties of working *in vivo*. Although it must be kept in mind that interactions between various cells types occur continuously under normal circumstances, monocultures of monocytes derived either as primary cultures harvested from blood or peritoneal fluid, or maintained as cells lines, can provide data regarding direct effects of treatments *in vitro*, while holding most variables constant. THP-1 is a human leukemic cell line with a normal diploid karyotype (29) that was cultured initially from a boy with acute monocytic leukemia (90). These cells express surface markers and respond similarly to human monocytes, and can be maintained in monoculture as a nonadherent suspension with a doubling time of 60 – 70 hours. They can be differentiated to a more macrophage-like phenotype by treating them with various agents, including phorbol myristate acetate (PMA), vitamin D3, or retinoic acid. Differentiated THP-1 cells behave more like monocyte-derived macrophages than other human myeloid cell lines, including HL-60, U937, KG-1, or HEL cells (4). THP-1 cells must be maintained between $2 - 5 \times 10^5$ cells/ml and not used after approximately 20 passages due to losses in responsiveness. Most of the work for this thesis was done using THP-1 cells, but comparative studies were done on murine resident peritoneal

macrophages harvested from Balb/C (Jackson Labs) mice, and RAW 264.7 cells. RAW 264.7 cells (ATCC) are virally-transformed mouse peritoneal macrophages that form adherent monolayers in cell culture. They are well characterized as excellent models for the study of murine macrophage activation (43,96). They were, however, of limited use in studying arachidonic acid metabolism due to their constitutively high levels of arachidonic acid release.

There are very few good macrophage models, particularly for human cells. THP-1 cells were developed as a model for differentiation and expression of monocytic surface markers, but few studies have been done regarding their feasibility as a model for inflammation and particularly for lipid metabolism. My objective for the studies presented in this chapter was to characterize this cell line as a model for macrophage activation, appropriate for studies focusing on arachidonic acid metabolism. This included four specific aims. The first was to study basic growth characteristics and determine ideal growth conditions in order to get the most consistent results. Second, viability and morphological changes following treatments were observed using trypan blue exclusion and microscopy to determine that cell loss due to cell death or adherence would not significantly impact results. Third, we measured lipid composition and pool labeling by thin layer chromatography to demonstrate that treatments did not cause alterations of these parameters that would impact assay results. Finally, we measured the surface expression of CD14 using flow cytometry to determine whether treatments affected expression of this LPS receptor.

MATERIALS AND METHODS

Amphiphiles and reagents. All LPS and lipid A preparations were provided by Ribi ImmunoChem Research (Hamilton, MT). Stock solutions of amphiphiles at 1000 μg/ml were kept at 4° C in 0.1% triethylamine (TEA). LTA from *E. faecalis*, PMA, dexamethasone, N-acetylceramide, phospholipid and sphingolipid standards for thin layer chromatography, and 2-mercaptoethanol were obtained from Sigma. Vitamin D3 (1α, 25 dihydroxyvitamin D) was purchased from Calbiochem (LaJolla Ca). [^{3}H]-arachidonic acid was purchased from American Radiolabeled Chemical Co. (St. Louis, MO). Minicolumns for lipid separations were purchased from Amersham. Monoclonal antibodies for flow cytometry were from Becton-Dickinson Immunocytometry Systems (San Jose CA) and Coulter Laboratories.

Cell Culture. THP-1 cells from ATCC were cultured in RPMI from Mediatech, supplemented with 10% Hyclone fetal bovine serum (FBS) and 2 x 10^{-5} M 2-mercaptoethanol at 37° C in a 5% CO_2 incubator. Cells were maintained between 2.5 x 10^5 and 1 x 10^6 cells/ml. Prior to experiments, cells were incubated overnight with a maturation agent, either 10^{-7} M vitamin D3 or 2 nM PMA. Although loosely adherent, cells could be removed by pipeting or gentle scraping.

Lipid analysis. Lipids were extracted from THP-1 cells by a modification of the Bligh and Dyer procedure (12). THP-1 cells were grown in either ^{32}P or ^{3}H-AA (0.5 μCi/ml) to label the lipid pools. Cells were suspended in 1 ml cold NaCl (0.3%) and then heated at 68° C in an additional 3 ml of solvent containing chloroform:methanol:1 N HCl

(1:2:0.3 v/v) for 5 min. The cooled suspension was centrifuged and the supernatant (lipid extract) was transferred to a clean centrifuge tube The cell residue (pellet) was extracted again with 1.9 ml of solvent containing chloroform:methanol: 0.3% NaCl (1:2:0.8 v/v). The combined extracts were mixed thoroughly with 1.5 ml chloroform and 1.5 ml 0.3% NaCl. This mixture of chloroform:methanol:water (2:2:1.8 v/v) provided a two phase system that was separated by centrifugation. The chloroform phase was removed and taken to dryness, and resuspended in chloroform:methanol (2:1 v/v). Total lipid extracts were analyzed by thin layer chromatography on Whatman LD6D plates using a solvent system consisting of chloroform-ethanol-water-diethylamine (30:34:8:35 v/v). To quantify the lipids (^{32}P-labeled phospholipids, or ^{3}H-AA) in each band, the bands were scraped from the plates and counted in a liquid scintillation spectrometer.

Lipids released from activated cells were also separated into prostaglandin, peptidoleukotriene, and fatty acid fractions on Amprep C18 minicolumns (Amersham) using the following solvent system: (solvent A); acetonitrile-methanol-water-acetic acid (8:5:12:0.025 v/v). After washing the column with water, prostaglandin E_2 and related compounds were eluted with solvent A-water (75:25), peptidoleukotrienes with solvent A-methanol (50:50), and arachidonic acid with methanol.

Challenge experiments

Eicosanoid release. Differentiated THP-1 cells were labeled overnight with 0.5 μC/ml ^{3}H-AA (specific activity = 200 Ci/mmole) in RPMI with 10% FBS, then washed three times with warmed RPMI. Cells were then gently removed from flasks and plated in 24-well plates at approximately 1.5 x 10^{6} cells/well in 0.5 ml RPMI. Challenge

concentrations (2x) were added, 0.5 ml/well in RPMI with 4% FBS. Plates were incubated for 2 hr at 37° C. Supernatants were removed to microfuge tubes and spun at 3000 rpm for 3 min to remove any cells. 200 µl of the cell-free supernatants were counted in a Beckman scintillation spectrometer. For uptake experiments, 200 µl of supernatants before challenge (counts not taken up), and 200 µl of cell lysates (1 ml 1% Triton X100) from each sample (uptake) were also counted.

Cytokine release. Differentiated THP-1 cells were plated in 24-well plates at 5 x 10^5 cells/ml in 1 ml RPMI with 5% FBS. Cells were challenged for 16 hr at 37° C. Supernatants were collected and stored at –70° C until assayed with ELISA kits for cytokines released. Amounts released in pg/ml were determined from a standard curve.

Flow Cytometry. After overnight treatment with D3, ceramide (C2), or LPS, THP-1 cells were stained with FITC or phycoerythrin (PE) conjugated monoclonal antibodies to CD14 epitopes including anti-CD14/FITC (MØ-P9) from Becton-Dickinson and anti-CD14/PE (MY-4) from Coulter Labs. PE-conjugated monoclonal antibodies to constitutively-expressed leukocyte marker, CD45, were used as a positive control stain to demonstrate consistent staining of samples. The staining period was 30 min at 4°C. Cells were washed twice with staining buffer (PBS, 0.05% sodium azide, 2% FBS) and fixed with 1% buffered paraformaldehyde. PE and FITC conjugated isotype-matched negative control antibodies to myeloma proteins were used throughout to quantitate nonspecific background binding to target cells. Forward angle (FALS) and 90° (orthogonal) light scatter gating eliminated debris and dead cells from the analysis on a

Coulter elite flow cytometer. Data represent the percentage of positively stained cells for a given antibody, projected beyond the cursors set on negative control histograms to separate positively and negatively stained cells. Cursors were set so that 1-2% of all cells stained with the negative control, isotype-matched reagents were displayed as being in the positive region of the histogram.

RESULTS

Growth, viability, and adherence. THP-1 cells growing between 2 – 6 x 10^5 cells/ml in RPMI with 10% FBS show a doubling time of 72 hours (Fig. 1) and maintain a viability of 98% by trypan blue exclusion. Treatment with 0.1 μg/ml LPS R595, 0.1 μg/ml LTA (*E. faecalis)*, or 0.1 μM vitamin D3 for 24 hours caused temporary or partial adherence (Fig. 2), though cells could be gently removed from culture flasks by pipeting. PMA (30 ng/ml) caused significant differentiation within 24 hours to macrophage-like morphology, including adherence, spreading, and increases in granular inclusions. N-acetylceramide (C2 ceramide), a short-chain, cell-permeable ceramide, did not induce adherence or morphological changes. Viability remained above 95% with all of these treatments except for PMA, which dropped viability to 91% by 72 hours.

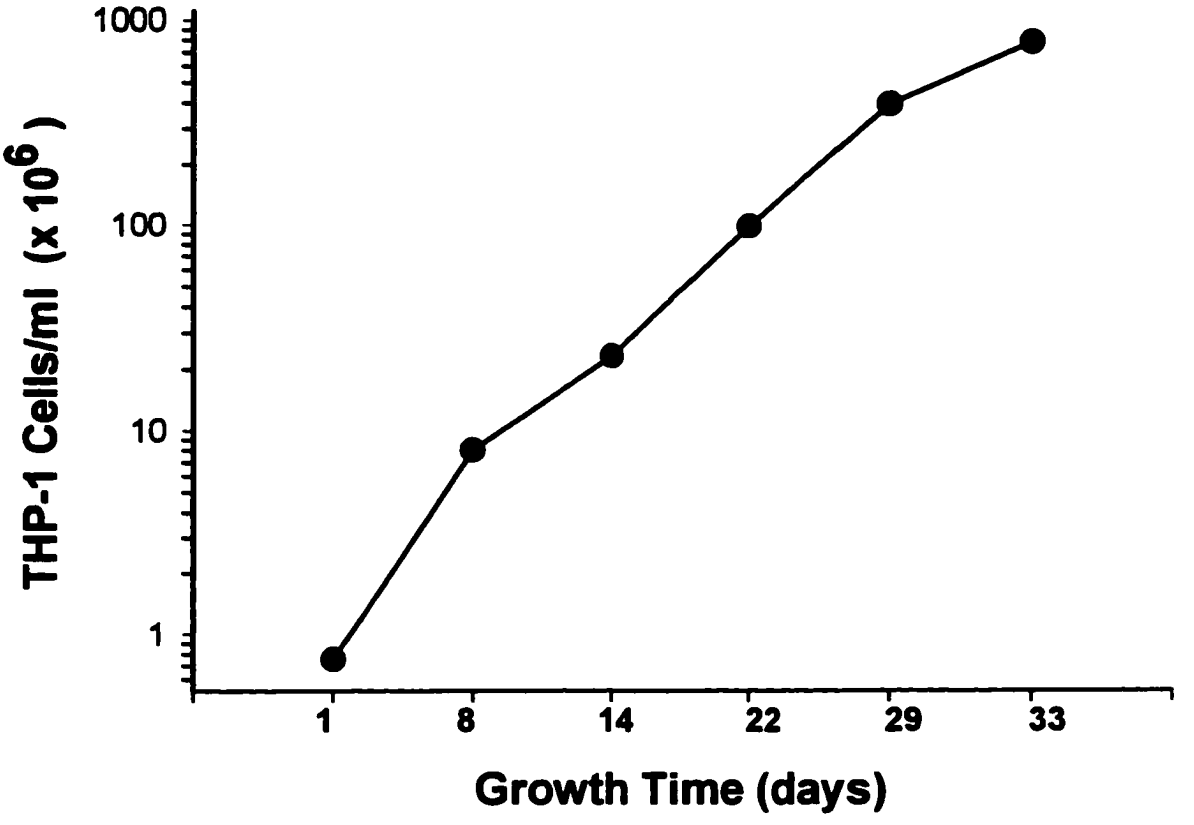


Figure 1. THP-1 Growth Curve. THP-1 cells were maintained at 0.3-1x10^6 cells/ml in RPMI with 10% PBS at 37°C in 5% CO_2. Cells were counted by hemocytometer, and cells/ml were calculated to include dilutions performed when cells were fed and split every 3 days. Viability was determined to be ≥ 98% by trypan blue exclusion. A typical experiment is shown.

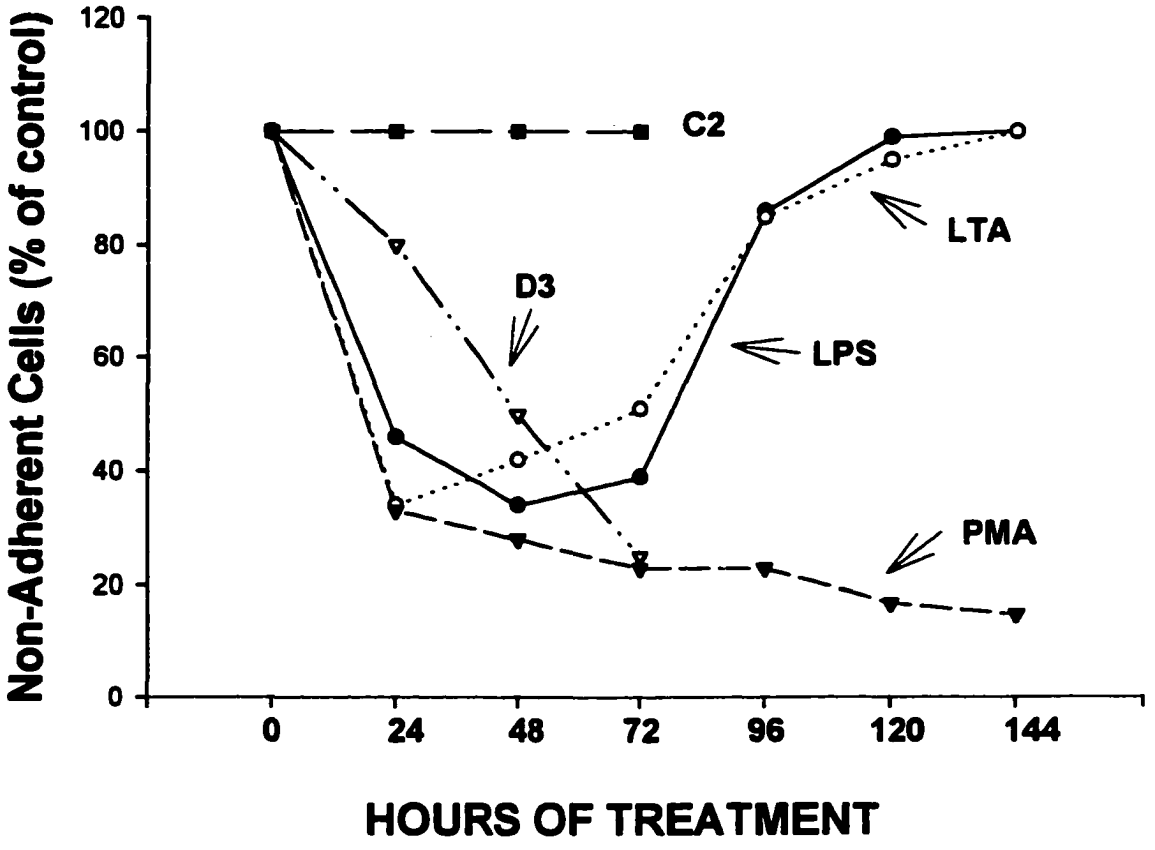


Figure 2. Induction of adherence in THP-1 cells by various treatments. THP-1 cells were grown in 24-well plates in 1 ml RPMI with 10% FBS, starting at $5x10^5$ cells/ml. Treatments were added at time 0 when all cells were in suspension. The percent of non-adherent cells was determined every 24 hours by counting non-adherent cells by hemocytometer, then scraping the wells and counting total cells/ml. Treatments were at the following concentrations: 0.1 μg/ml LPS (R595), 0.1 μg/ml LTA (*E. faecalis)*, 30 ng/ml PMA, 0.1 μM vitamin D3, and 60 μM C2 ceramide. Viabilities remained above 91% throughout the experiment.

Lipid composition and distribution of label in lipid pools. THP-1 cells were grown for 72 hours in medium containing 0.5 μCi/ml ^{32}P (added as carrier-free $H_3{}^{32}PO_4$). Under these conditions, the specific activity of phosphorus (cpm/nmol phosphorus) in each lipid fraction was identical to the specific activity of the total cellular phosphorus. Total lipids were extracted from these cells as described in Materials and Methods, and the lipids were fractionated by thin-layer chromatography using two solvent systems. Individual lipids were located by autoradiography and identified by staining and co-chromatography with known standards (Fig. 3). Table 1 summarizes the major phospholipids in THP-1 cells before and after a 4 hr treatment with LPS (R595 @ 100 ng/ml). The primary phospholipid was phosphatidylcholine (PC) in both cases, but treatment with LPS resulted in a slight, and transient, decrease in the PC pool.

TABLE 1. Phospholipid composition of THP-1 cells.[a]

	Percentage of Total Lipid[b]				
	SM	PC	PI	PE	PA & NL
Control	3	53	13	27	6
LPS	3	49	15	27	6

a) Lipids were separated on Whatman LK6D silica gel plates using a solvent system consisting of chloroform-methanol-acetic acid 65:25:8.

b) SM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PA, phosphatidic acid; NL, neutral lipids (unidentified). Experiment was run in quadruplicate, SD<1%.

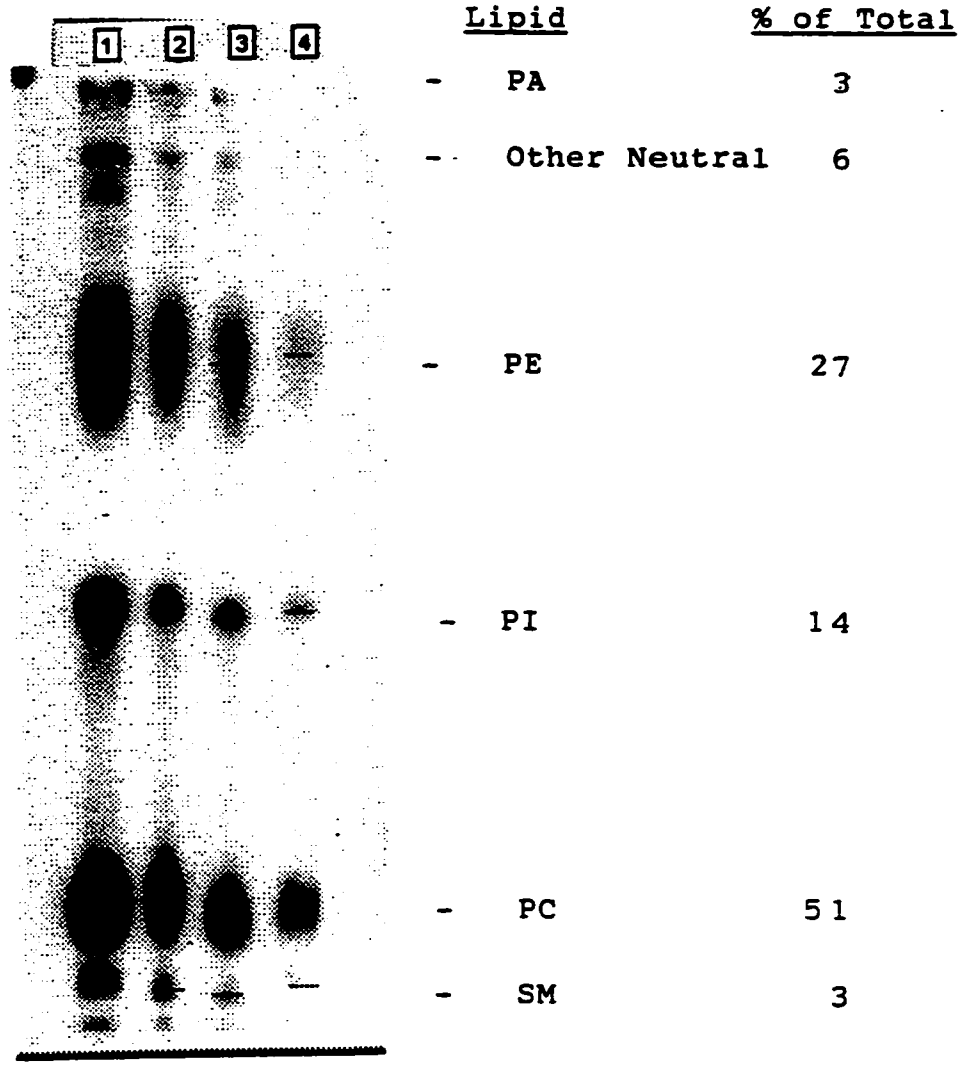
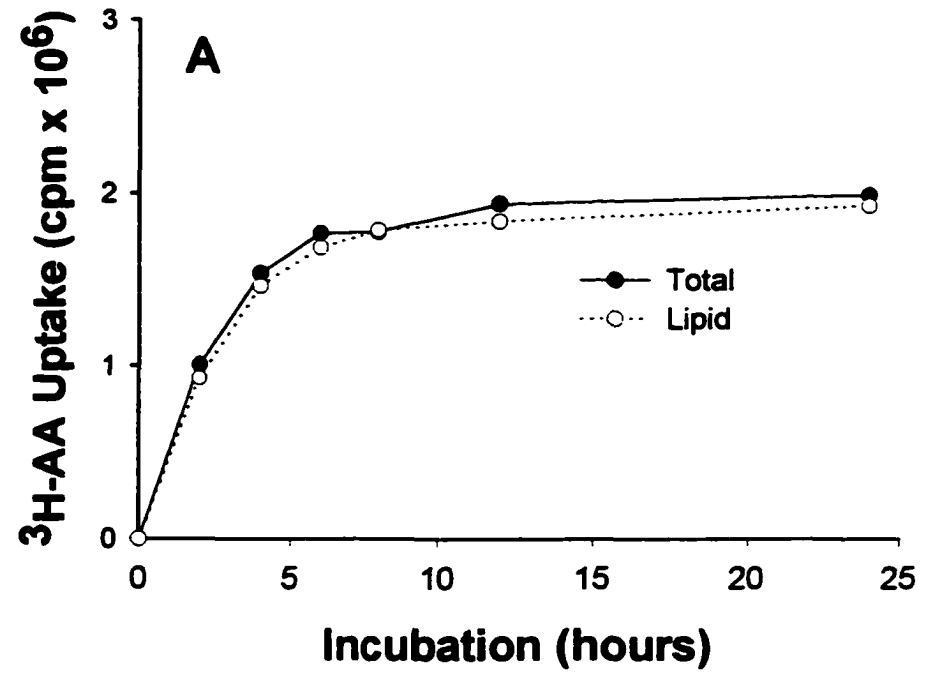
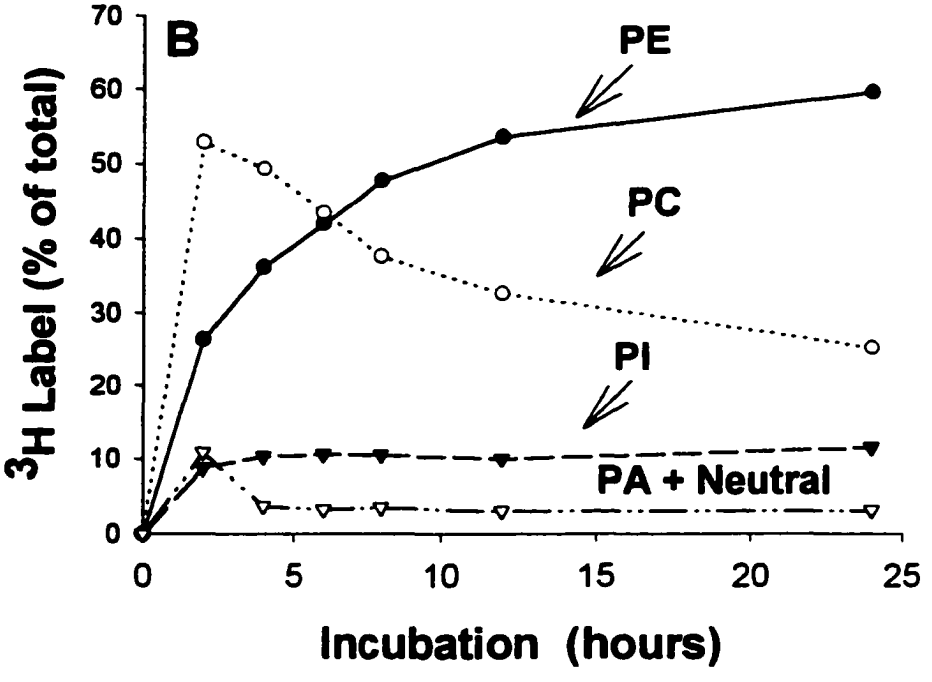


Figure 3. Chromatograph of ^{32}P- phospholipids in THP-1 cells. Lipid extracts of THP-1 cells grown in medium containing ^{32}P were separated by TLC, as described in Materials and Methods. Lanes 1-4 are simply varying amounts of extract spotted on the plate, from 15µl – 2.5 µl. The bands were scraped from the plates and counted for cpm released. Bands were identified by lipid standards. Values represent the counts for each lipid as a percent of the total. Experiments run in quadruplicate, SD<1%.

As discussed in Chapter 1, the release of eicosanoids is an important index of macrophage activation, and is determined by measuring the amount of tritium released from cells labeled with ^{3}H-arachidonic acid. Cells were routinely labeled overnight (18 to 24 hours) prior to challenge. In order to determine the distribution of label in the lipid pools, THP-1 cells were treated with 0.1 μM vitamin D3, and labeled with 0.5 μCi/ml ^{3}H-AA overnight. Samples were removed at intervals, mixed with unlabeled carrier cells, suspended in cold HBS and centrifuged. The amount of label taken up by the cells was determined by counting an aliquot of the sample before and after removing the cells by centrifugation. The cells were washed twice with cold HBS, then suspended in 0.3% NaCl and extracted as described in Materials and Methods. All of the ^{3}H taken up by THP-1 cells was incorporated into the lipid pools (Fig. 4A), which suggests that little, if any, degradation of arachidonic acid prior to incorporation into the lipid pool occurred under these conditions. Figure 4B shows that label initially appeared in the PC and neutral lipid pools. These results suggest that PC is the most metabolically active lipid and that the label which is initially incorporated into the PC pool is subsequently chased into the PE pool. This pattern of metabolism is consistent with the hydrolysis of PC to diglyceride by a PC-specific phospholipase C and the subsequent conversion to both PC and PE.

Figure 4. Distribution of tritium in the lipid pools. A) Kinetics of incorporation of label into THP-1 cells over 24 hours. The cells were cultured and extracted as described in the text and Materials & Methods. Values show the cpm from whole cells and their total lipid extracts. **B)** Kinetics of uptake of ^{3}H-AA into major phospholipid pools. Values for each lipid show percentages of the total cpm in the lipid pool, and represent quadruplicate samples with SD<1%.





Treatment of THP-1 cells with vitamin D3 did increase the overall uptake of ^{3}H-AA slightly. Vitamin D3 treated cells contained 3 x 10^5 cpm/10^6 cells (2.1 x 10^6 cpm/mg protein) whereas untreated cells contained 2.1 x 10^5 cpm/10^6 cells (1.3 x 10^6 cpm/mg protein). However, none of the challenge treatments tested significantly altered overall uptake of ^{3}H-AA (Fig.5).

Once incorporated into the lipid pools, ^{3}H-AA can be released by activation of PLA_2 by various challenges. The arachidonic acid can then be metabolized by specific enzymes. However, of the labeled lipid released, the major component was free arachidonic acid (Fig. 6). To show that, after challenge, most of the arachidonic acid was released via PLA_2 activity as suggested in the literature (27,33), we used PLA_2 inhibitor dexamethasone to block release of ^{3}H-AA (Fig. 7). It is assumed that the constitutive release of ^{3}H in the supernatant is due to turnover by other enzymes or by a PLA_2 which is not affected by dexamethasone.

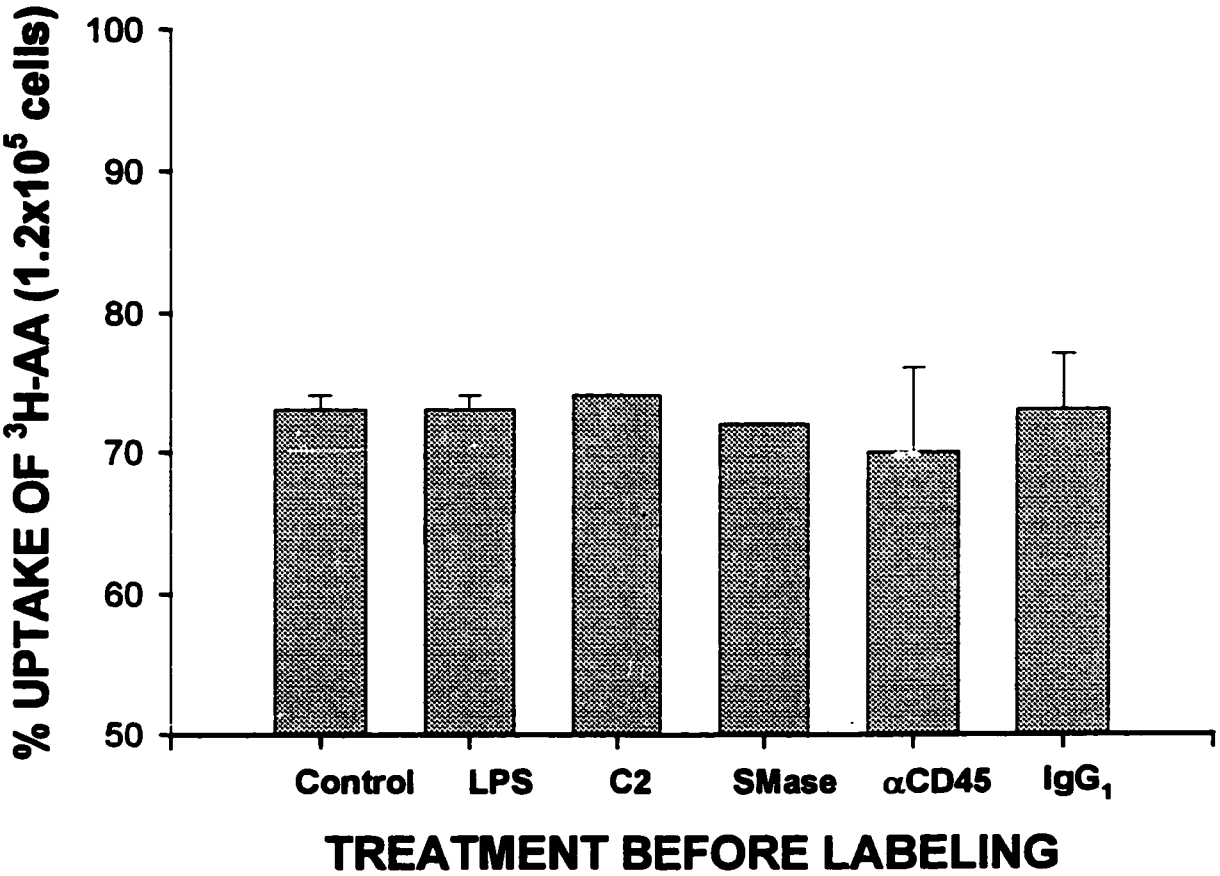


Figure 5. Uptake of ^{3}H-AA after treatment. THP-1 cells at 6x10^5/ml were pretreated with 0.1 μM vitamin D3 for 24 hr, and then treated for 3 hr with 100 ng/ml LPS (R595), 50 μM C2 ceramide, 0.25 U/ml Smase (*S.aureus*), 20 μl/ml αCD45 2D1, or 20 μl IgG$_1$ (isotype matched control). The cells were washed, resuspended in fresh medium, and labeled with 0.5 μci/ml ^{3}H-AA overnight. Cells were removed by centrifugation, and 200 μl of supernatant were counted. Cell pellets were lysed by freezing in 1 ml 1% Triton x100, and 200 μl of the lysate were counted. Uptake was calculated as a percent of the total cpm for each sample. Values are averages of duplicates ± range.

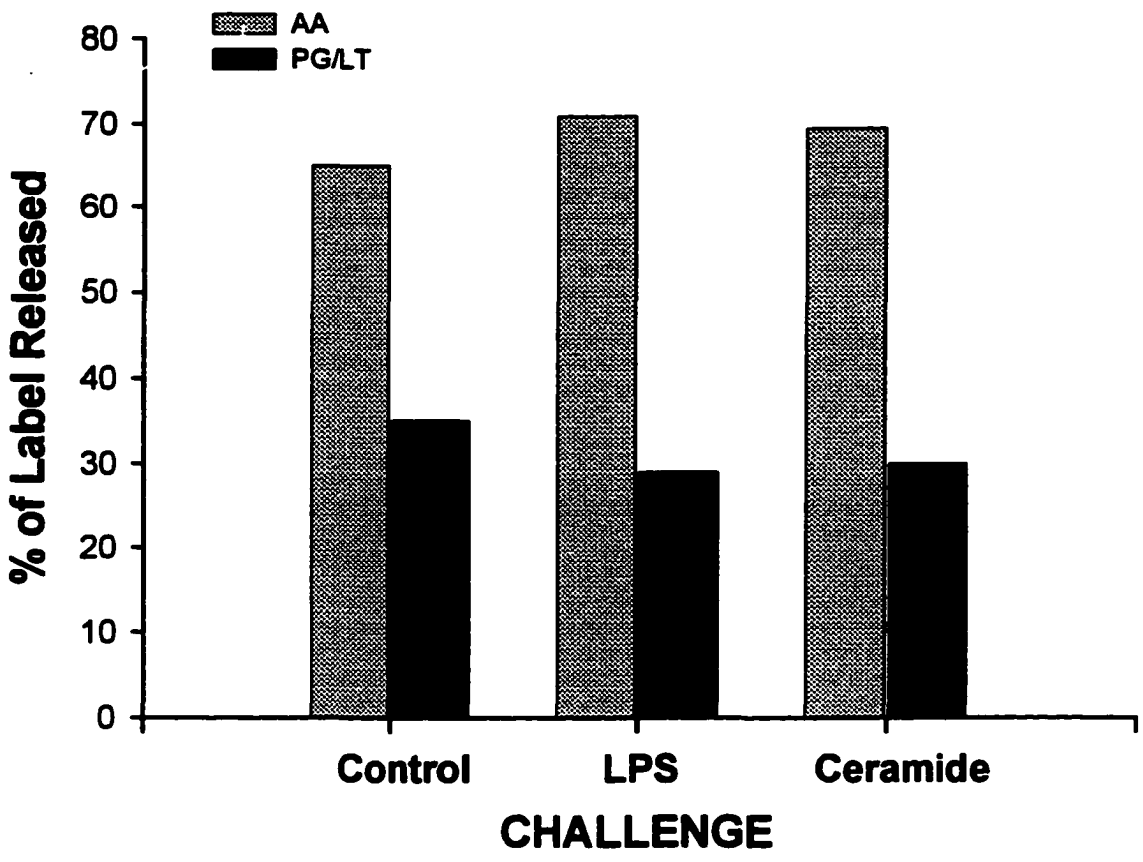


Figure 6. Labeled eicosanoids released after challenge. THP-1 cells were pretreated, and then labeled overnight as in Fig. 4. Cells were washed and resuspended in fresh medium alone, or with 0.1 μg/ml LPS (R595) or 80 μM C2 ceramide for 3 hr. Cells were removed by centrifugation, and lipids were extracted from the supernatants as described in Materials and Methods. Lipids were separated into prostaglandin, peptidoleukotriene, and fatty acid fractions on Amprep C18 minicolumns. Values similar to those shown here were seen in > 3 experiments.

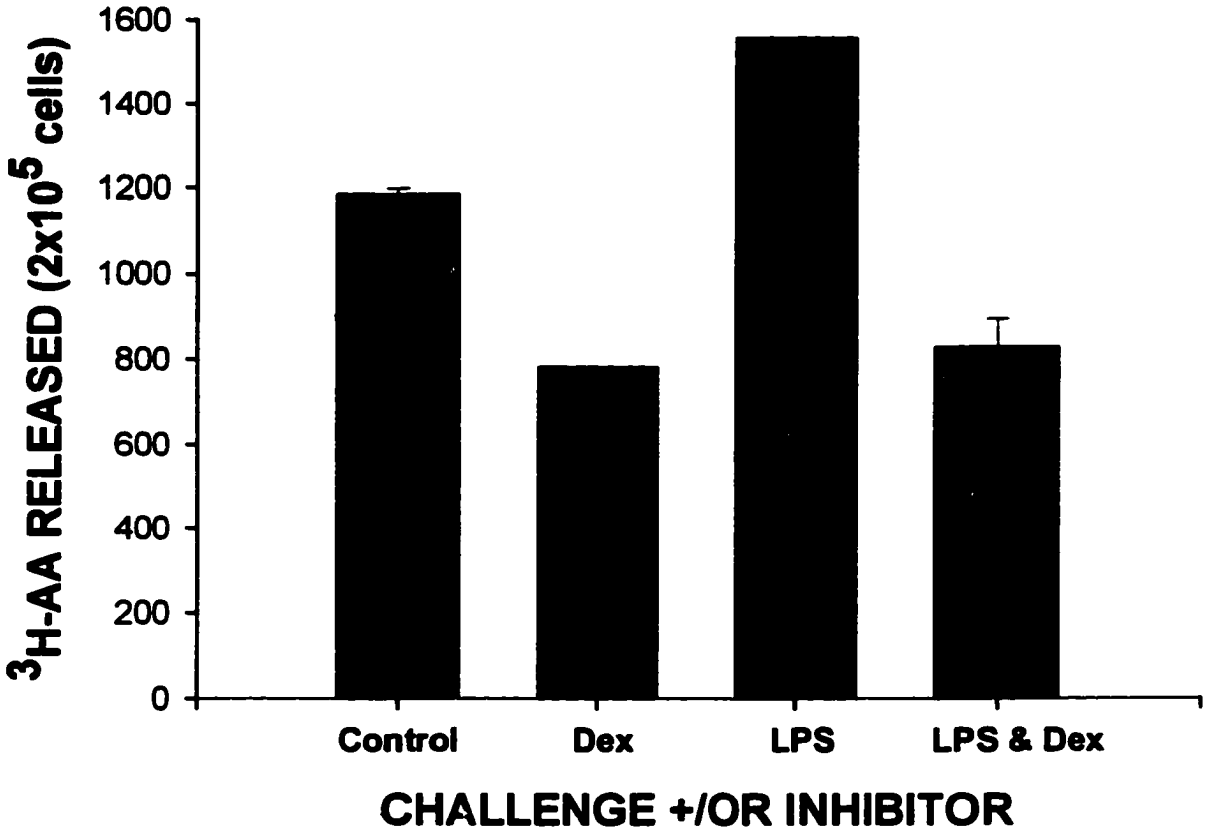
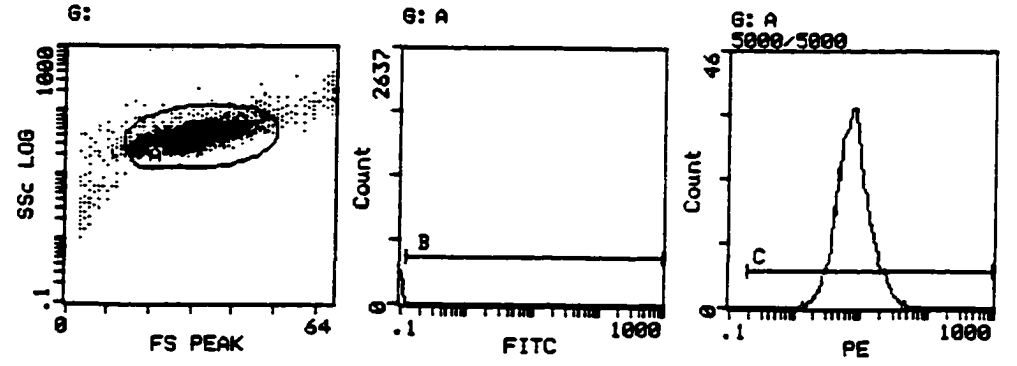


Figure 7. Inhibition of ^{3}H-AA release by dexamethasone. THP-1 cells were labeled as in Fig. 5, washed and resuspended in fresh medium alone, medium plus 20 μM dexamethasone (Dex), medium plus 100 ng/ml LPS (R595), or medium plus Dex and LPS. After 3 hr, cells were removed by centrifugation and 200 μl of supernatants were counted for cpm released. Values are averages of duplicates ± range.

CD14 expression. THP-1 cells can be differentiated to a more mature macrophage-like phenotype with 1α,25 dihydroxyvitamin D (vitamin D3). We found that a 24 hour treatment with 0.1 μM D3 would induce maximal expression of CD14, while leaving the cells only slightly adherent and easy to remove from culture flasks. THP-1 cells were analyzed by flow cytometry for expression of CD14 using two different monoclonal antibodies: MØ-P9, which does not block LPS stimulation of THP-1 cells, and clone MY-4, which does block LPS stimulation (unpublished data, Ribi ImmunoChem). The histograms in Figure 8 show the increase in expression of CD14 MØ-P9 following treatment with vitamin D3. Both the percentage of cells staining positive in the gated population (the histogram peak), and the mean channel fluorescence (location of the peak on the X axis) increased by at least 50% for both anti-CD14 antibodies within 18 hours after treatment with D3. However, neither % positive nor mean channel fluorescence was significantly altered by LPS, MLA, or ceramide (Table 2).

Consistent with our findings, treatment with vitamin D3 has been reported to increase sensitivity of THP-1 cells to LPS (57,69,98), presumably due to the expression of CD14. Figure 9 shows that pretreatment with vitamin D3 also increased THP-1 cell sensitivity to ceramide for the release of arachidonic acid metabolites. It is unlikely that expression of CD14 is involved in ceramide signaling, but it is very possible that D3 up-regulates other signal components that have not yet been determined.

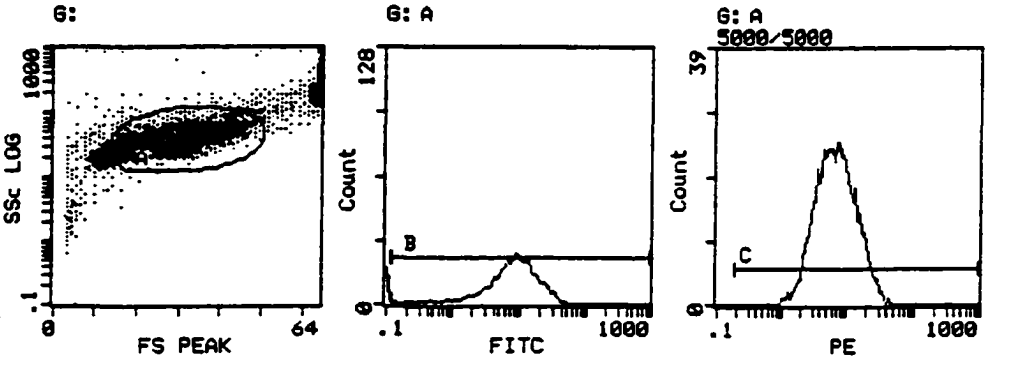


SINGLE PARAMETER STATISTICS

ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	SD	FullCV	HalfCV	Min	Max
B	40.8	2041	0.15	14	0.665	0.924	138.9	0.827	0.12	1024
C	100	5000	8.4	46	7.76	4.54	58.6	24.2	0.19	1024

DUAL PARAMETER STATISTICS

ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	X Channel SD	X Channel CV	Y Channel Mean	Y Channel SD	Y Channel CV
A	85.4	5000	29, 32	67	29.8	7.9	26.4	36.7	13.6	37.1



SINGLE PARAMETER STATISTICS

ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	SD	FullCV	HalfCV	Min	Max
B	96.8	4840	12	34	7.20	7.37	102.4	1.47	0.12	1024
C	99.9	4994	8.8	39	6.69	4.31	71.9	3.39	0.19	1024

DUAL PARAMETER STATISTICS

ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	X Channel SD	X Channel CV	Y Channel Mean	Y Channel SD	Y Channel CV
A	65.5	5000	29, 28	46	29.6	8.7	29.5	37.7	16.1	42.9

Figure 8. Demonstration by flow cytometry of increased expression of CD14 after D3 treatment. THP-1 cells, untreated (top) or treated (bottom) with vitamin D3 for 18 hours, were analyzed for expression of CD14 using anti-CD14 MØ-P9. Cells were stained and analyzed by flow cytometry as described in Materials and Methods. A) Gated population, B) anti-CD14/FITC, C) anti-CD45/PE (Positive control).

TABLE 2

Surface expression of CD14 measured by flow cytometry after treatment with LPS, C2 ceramide, or vitamin D3.[a]

TREATMENT:	Control	LPS	C2	MLA	D3
αCD14(MØ-P9)	5.9[b]	4.9	5.5	3.9	82.2
	8.4[c]	7.1	7.6	7.2	15.4
αCD14 (MY-4)	52.5	67.5	56.7	63.1	97.8
	4.4	4.4	4.0	4.0	52.5
αCD45 (2D1)	98.8	97.8	98.8	98.9	95.7
	14.6	15.6	12.5	16.5	9.5

a) THP-1 cells were treated for 18 hr with medium only, 100 ng/ml LPS (R595), 40 μM C2 ceramide, 100 ng/ml MLA, or 0.1 μM vitamin D3. Cells were then stained and analyzed by flow cytometry as in Materials and Methods.

b) Percent of gated (90% vs FALS) cells staining positive

c) Mean channel fluorescence – 4 decade logarithmic range

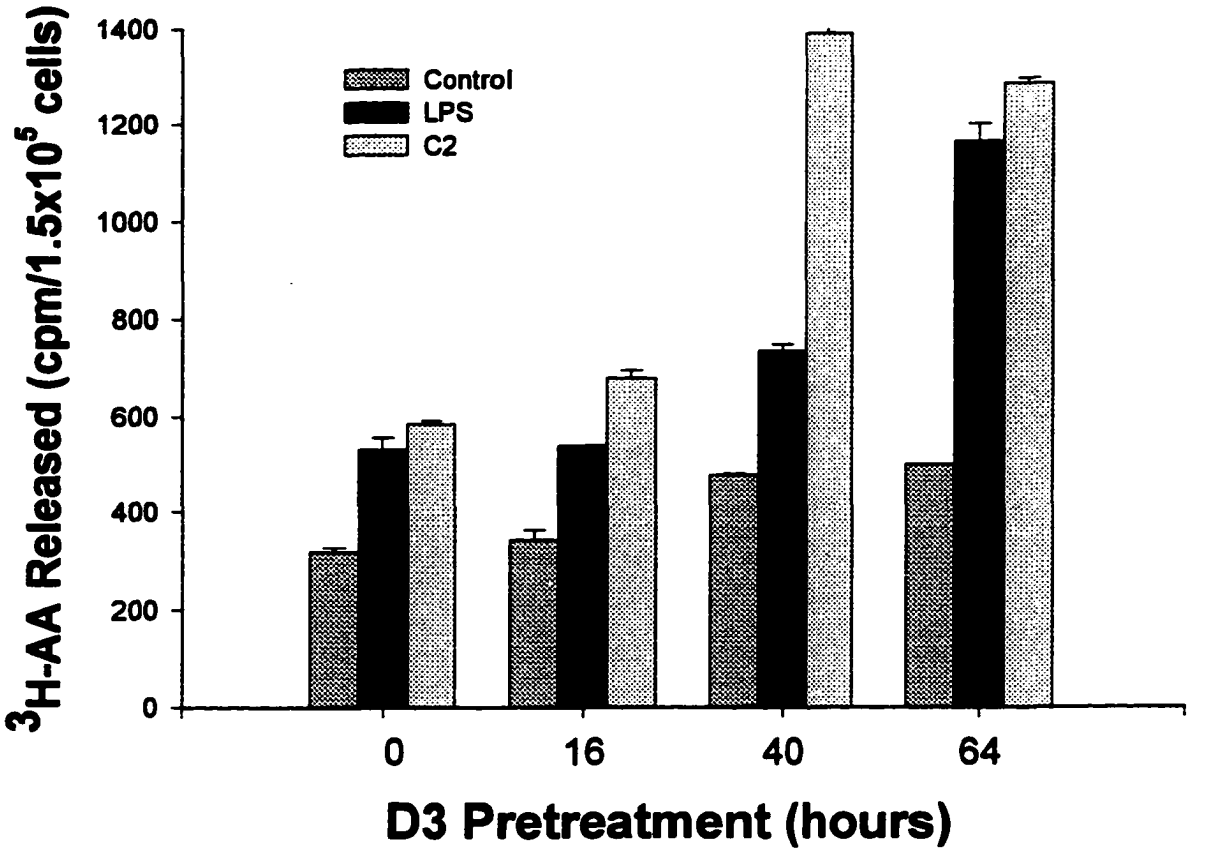


Figure 9. Increased sensitivity of THP-1 cells after D3 pretreatment. THP-1 cells were treated with 0.1 μM vitamin D3 for the indicated times, labeled with ^{3}H-AA and challenged with medium only, 1 μg/ml LPS (R595), or 100 μM C2 ceramide for 4 hr. 200 μl of post-challenge supernatants were counted. Values are averages of duplicate samples ± range, and results are representative of 2 experiments.

DISCUSSION AND CONCLUSION

THP-1 cells have proven to be a good model for our studies as long as they are cultured at consistently low density (2 - 6 x 10^5 cells/ml) and maintained for about 20 passages before starting a fresh batch from frozen stocks. Vitamin D3 increased ^{3}H-AA uptake, CD14 expression, and sensitivity to challenge, making them responsive for our assays (Fig 8 & 9, Table 2). Studies have shown that in THP-1 cells, LPS induced hydrolysis of phosphatidylcholine (PC), but did not affect lipid pool labeling or the hydrolysis of phosphatidylethanolamine (PE) or phosphatidylinositol (PI) (18). We wanted to confirm this result to provide evidence that our treatments did not affect pool labeling. Because PC is the major substrate for PLA_2, altered pool labeling would confuse our results. Analysis of lipid extracts confirmed that the major phospholipids in THP-1 cells are PC and PE, and that these pools contained the majority of label following incubation with ^{3}H-AA (Figs. 3 & 4). Label in the PE and PI fractions remained constant or increased slightly after 10 – 15 hours of incubation in the presence of label, whereas label in the PC pool dropped off. This suggests that the PC is the most active in terms of turn-over.

None of our challenge treatments affected viability, CD14 expression, or uptake of ^{3}H-AA (Figs 2 & 5, Table 2). Therefore, these parameters could be ruled out as factors that might have affected sensitivity of the cells to challenge, and therefore the cpm released from treated cells.

PMA acts as a diacylglycerol analog, activating cells downstream from receptor-mediated signaling. It was used as a negative control for experiments assuming a

receptor-dependent mechanism (Chapter 5). It was not used as a differentiation inducer due to the high degree of adherence it caused (Fig.2), the mild cytotoxicity seen, and the fact that it does not increase CD14 expression (27).

Since free arachidonic acid was the major component of total labeled lipid released (Fig. 6), changes in the metabolism of a portion of the free fatty acid to prostaglandins or leukotrienes might not be apparent from simply measuring ^{3}H-AA release. It was therefore important for the rest of the studies to check for different patterns of signaling induced by various challenges, by measuring specific eicosanoids. Inhibition of release with dexamethasone, a phospholipase A_2 inhibitor, showed that most of the arachidonic acid released from activated macrophages was due to PLA_2 activation (Fig. 7).

Most of the experiments for this thesis were performed using THP-1 cells. However, we frequently repeated tests using mouse peritoneal exudate cells (PEC) or RAW274.6 murine macrophages, and found that the mouse cells responded similarly in most cases. Experimental data demonstrating this are presented in subsequent chapters.

CHAPTER 3

ACTIVATION AND DESENSITIZATION OF MACROPHAGES BY BACTERIAL AMPHIPHILES

Introduction

Septic shock is the pathophysiological response to systemic infections by Gram negative or Gram positive bacteria. The response includes an exaggerated systemic inflammatory response, hypotension, intravascular clotting, multiple organ failure, and death. The biologically active molecules that appear to be involved in the activation of this response are amphiphiles located in the outer membrane or wall of these bacteria, including lipopolysaccharride (LPS) and lipoteichoic acids (LTA), respectively. In Gram negative septic shock, the majority of the toxicity is attributed to the lipid A component of LPS. LTA is 100 to 1000-fold less potent than LPS, and does not appear to be able to induce septic shock by itself. There is some evidence that septic shock caused by Gram positive bacteria results from the combined effects of LTA and peptidoglycan (22). Nevertheless, LTA has been shown to bind similar receptors as LPS (19,25,38), activate several common pathways (72), and is able to desensitize cells to subsequent challenge with LPS (16).

Although structurally different in many ways, both LPS and LTA are anionic polymers containing lipid, carbohydrate, and phosphorus. Intriguingly, both have also been shown to vary considerably in potency depending on the source organism (10,16,38,65), which suggests that minor variations in the molecular structure affect the

ability of the host to recognize and respond to the amphiphiles. Studies on the molecular and supra-molecular structure of many forms of LPS have shown that variations in acyl chain length and fluidity, acylation patterns, saccharide components, and phosphate residues all affect aggregate formation, and the toxicity and immunostimulatory effects of LPS preparations (14,40). Similarly, the charge ratios of the monomer, the acyl chains, and the size of the polyglycerophosphate chain all affect micelle formation of LTA and the binding of LTA to cellular receptors (38). This suggests that immune cells recognize and respond differentially not only to the molecules themselves, but to these unique aggregates, so that the signal transduction initiated at the cell surface varies to produce a wide range of responses. For example, when LPS from the rough mutant of *Salmonella minnesota* R595 is modified to monophosphoryl lipid A (MLA), toxicity is significantly reduced but the immunostimulatory activity is maintained (40). A relationship between structures of various LTAs and their potencies has not been found. In fact, two similar LTAs have been shown to have very different potencies in terms of activation of arachidonic acid metabolism (16).

The potent toxic and immunostimulatory properties of LPS and other bacterial amphiphiles result from lipid and protein mediators released primarily from activated macrophages. It is not clear whether these differential effects result from differences in the amount of all mediators released or from a selective increase or reduction of particular mediators. The phenomenon of desensitization also appears to occur just downstream from binding and does not appear to involve down-regulation of any LPS-binding surface proteins (28). The ability of LPS preparations to desensitize cells has

been considered a possible mechanism to exploit in the treatment and/or prevention of septic shock. Preliminary studies in animal models have shown that pretreatment with low levels of LPS, especially the less toxic forms, can be protective (3,40). However, *in vitro* studies are necessary to determine the mechanism of desensitization before the feasibility of further *in vivo* testing would be justified. Because the release of eicosanoids is critical in the development of inflammatory reactions, it was important to begin to characterize the release of arachidonic acid metabolites following treatment with various bacterial amphiphiles as a baseline for studies on pyrogenicity and the impact of monomeric and supramolecular structures on potency and toxicity. The preliminary work reported here has led to further investigations still underway through a contract with Ribi ImmunoChem Research.

In this study, we have compared the response of THP-1 cells to various bacterial amphiphiles, including LPS R595 and its derivatives, monophosphoryl lipid A (MLA) and diphosphoryl lipid A (DPL); smooth LPS from *S. abortus*, and LTA (*E. faecalis)* as measured by the release of arachidonic acid metabolites and pro-inflammatory cytokines. We also demonstrate the ability of both MLA and LTA to desensitize THP-1 cells to subsequent challenge with LPS, and this was correlated with the release of a putative autocrine factor, PGE_2. Experiments repeated using mouse peritoneal cells (PEC) or RAW264.7 murine macrophages showed similar results.

MATERIALS AND METHODS

Amphiphiles and reagents. All LPS and lipid A preparations were provided by Ribi ImmunoChem Research (Hamilton, MT). Stock solutions of amphiphiles at 1000 μg/ml were kept at 4° C in 0.1% TEA. Vitamin D3 (1α, 25 dihydroxyvitamin D) was purchased from Calbiochem (LaJolla CA). LTA from *S. faecalis*, PMA, and 2-mercaptoethanol were obtained from Sigma. Quantikine ELISA kits for tumor necrosis factor alpha (TNFα), IL-1, and IL-8 were purchased from R&D Systems, and EIA kits for eicosanoids were from Cayman Chemical (Ann Arbor MI). [^{3}H]-arachidonic acid was purchased from American Radiolabeled Chemical Co. (St. Louis, MO).

Cell Culture. THP-1 cells from ATCC were cultured in RPMI from Mediatech, supplemented with 10% Hyclone fetal bovine serum (FBS) and 2 x 10^{-5} M 2-mercaptoethanol at 37° C in a 5% CO_2 incubator. Cells were maintained between 2.5 x 10^5 and 1 x 10^6 cells/ml. Prior to experiments, cells were incubated overnight with a maturation agent, either 10^{-7} M vitamin D3 or 2nM PMA. Although loosely adherent, cells could be removed by pipeting or gentle scraping. RAW 264.7 cells from ATCC were maintained as adherent monolayers in Dulbecco's Modified Eagle's medium (Sigma) supplemented with 10% FBS. Peritoneal exudate cells were harvested by peritoneal lavage from Swiss Webster mice as previously described (16). After a 2 hour adherence period, non-adherent cells were removed by washing with warmed RPMI.

Challenge experiments

Eicosanoid release. RAW 264.7, PEC, or differentiated THP-1 cells were labeled overnight with 0.5 μC/ml ^{3}H-AA (specific activity = 200 Ci/mmole) in RPMI with 10%

FBS, then washed three times with warmed RPMI. Cells were then gently removed from flasks and plated in 24-well plates at approximately 1.5 x 10^6 cells/well in 0.5 ml RPMI. Challenge concentrations (2x) were added, 0.5 ml/well in RPMI with 4% FBS. Plates were incubated for 2 hr at 37° C. Supernatants were transferred to microfuge tubes and spun at 3000 rpm for 3 min to remove any cells. 200 µl of the cell-free supernatants were counted in a Beckman scintillation spectrometer. PG and LT fractions were determined by separation of radiolabeled lipids released from treated cells on C18 minicolumns using solvent systems discussed in Chapter 2 (Materials and Methods), or by EIA (Cayman Chemical).

Cytokine release. Differentiated THP-1 cells were plated in 24-well plates at 5 x 10^5 cells/ml in 1 ml RPMI with 5% FBS. Cells were challenged for 16 hr at 37° C. Supernatants were collected and stored at –70° C until assayed with ELISA kits for cytokines released. Amounts released in pg/ml were determined from a standard curve.

Desensitization experiments

Eicosanoid release. Cells were pretreated with MLA or LTA at various concentrations in medium with 10% FBS for 2 hr prior to the labeling period. Cells were washed twice with warm medium to remove pretreatment. Labeling and challenge periods were done as in challenge experiments.

TNFα assay. Cells were pretreated with MLA for 18 hr and were not washed prior to challenge. Cells were then challenged with LPS R595 at 10 ng/ml for 6 hr. Supernatants were assayed as in challenge experiments.

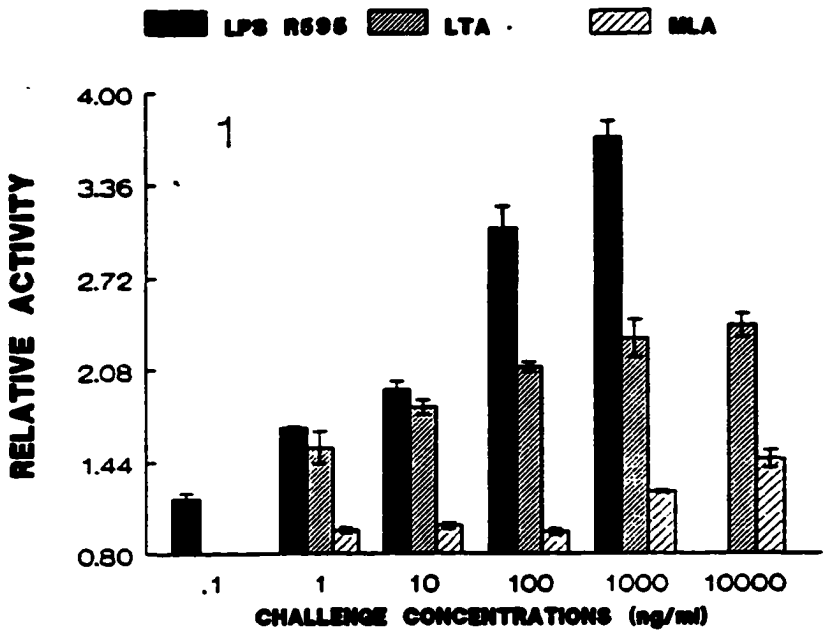
RESULTS

Arachidonic acid release from THP-1 and RAW264.7 cells by bacterial amphiphiles. The various challenges showed a range of potencies with respect to their ability to activate arachidonic acid metabolism in THP-1 cells, with the most dramatic difference between LPS R595 and MLA (Figs. 1 and 2). THP-1 cells required a thousand-fold higher concentration of MLA to release the amount of eicosanoids released by LPS R595. A 1000-fold higher concentration of MLA caused only 50% of the levels of cytokines released by LPS R595 (Fig. 3).

Figure 4 shows a significant difference in the potencies of two MLA preparations, MPL (MLA solubilized in 1% TEA) and MPL-s (MLA solubilized in 10% ethanol), with MPL-s approximately 70% more active for release of arachidonic acid.

In RAW264.7 murine macrophage cells, LPS R595 and *E. faecalis* LTA showed similar potencies (Fig. 5).

Prostaglandin/Leukotriene molar ratios released after challenge. Of the eicosanoids assayed, LPS, LTA and MLA all induced primarily prostaglandins, resulting in relatively high PG/LT ratios compared to amounts released from untreated cells (Fig. 6). In the experiment shown, the ratio of PGE_2/LTC_4 from MPL-s is considerably higher than the other amphiphiles. In other experiments, this ratio tended to slightly higher than the others, but not as dramatic as shown here.



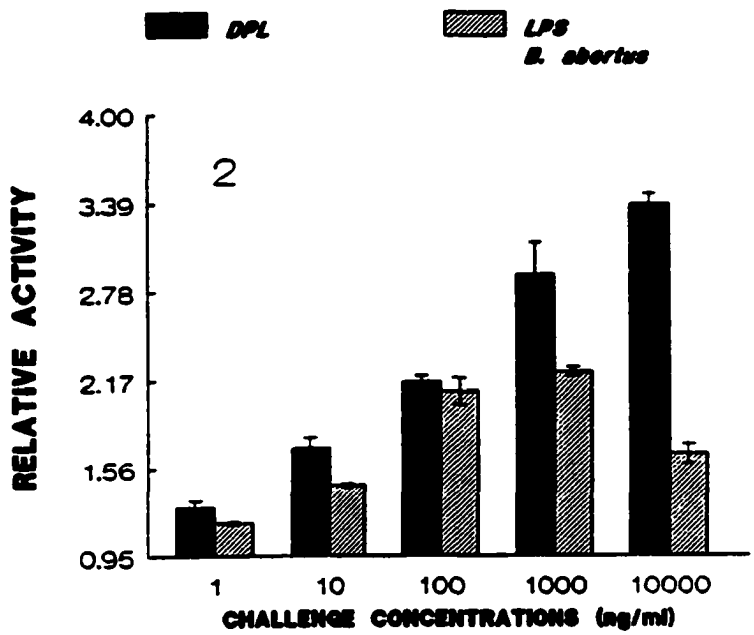


Figure 1 and 2. The effect of bacterial amphiphiles on ^{3}H-AA release. Differentiated THP-1 cells were labeled overnight with 0.5 μC/ml ^{3}H-arachidonic acid in RPMI with 10% FBS. Cells were washed and plated in a 24-well plate at $1.5x10^{6}$ cells/ml and challenged with concentrations of LPS R595, MLA, DPL, LPS (*S. abortus*), and LTA (*E .faecalis*) for 2 hours. 200 μl of supernatants were counted for cpm released. Relative activities were calculated based on labeled metabolites released relative to cells-only control. Points are averages of duplicates.

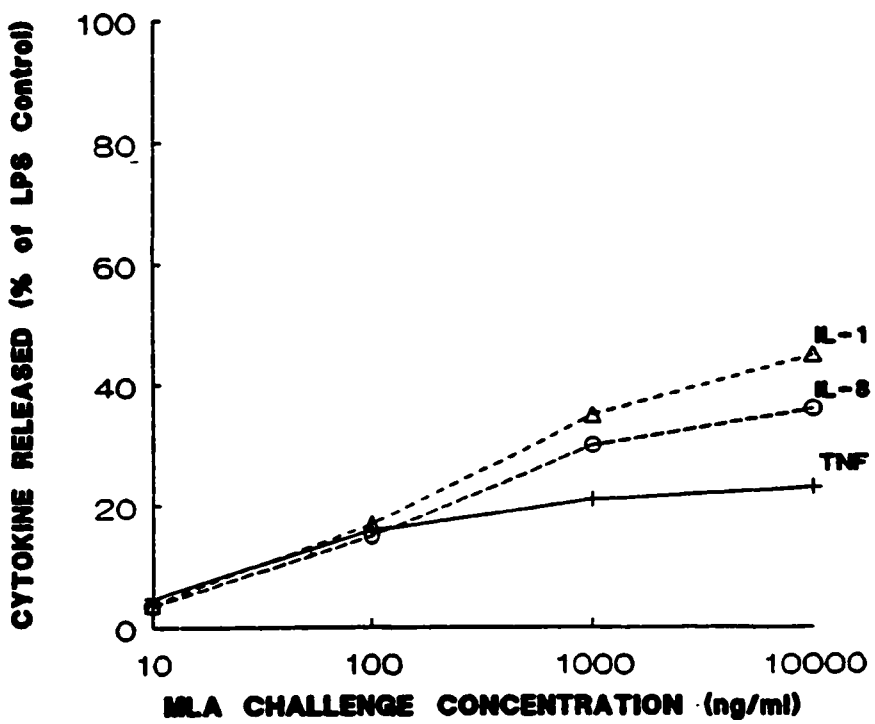


Figure 3. Effect of bacterial amphiphiles on cytokine release. Differentiated THP-1 cells were plated in 24-well plates at $1x10^6$ cells/ml and challenged with LPS R595 and MLA in RPMI with 5% FBS for 18 hours. Cytokines released were measured by ELISA and converted to pg/ml from a standard curve. Values represent amounts released by MLA as percents of the amount released by LPS R595 at 10 ng/ml. Points are averages of duplicates.

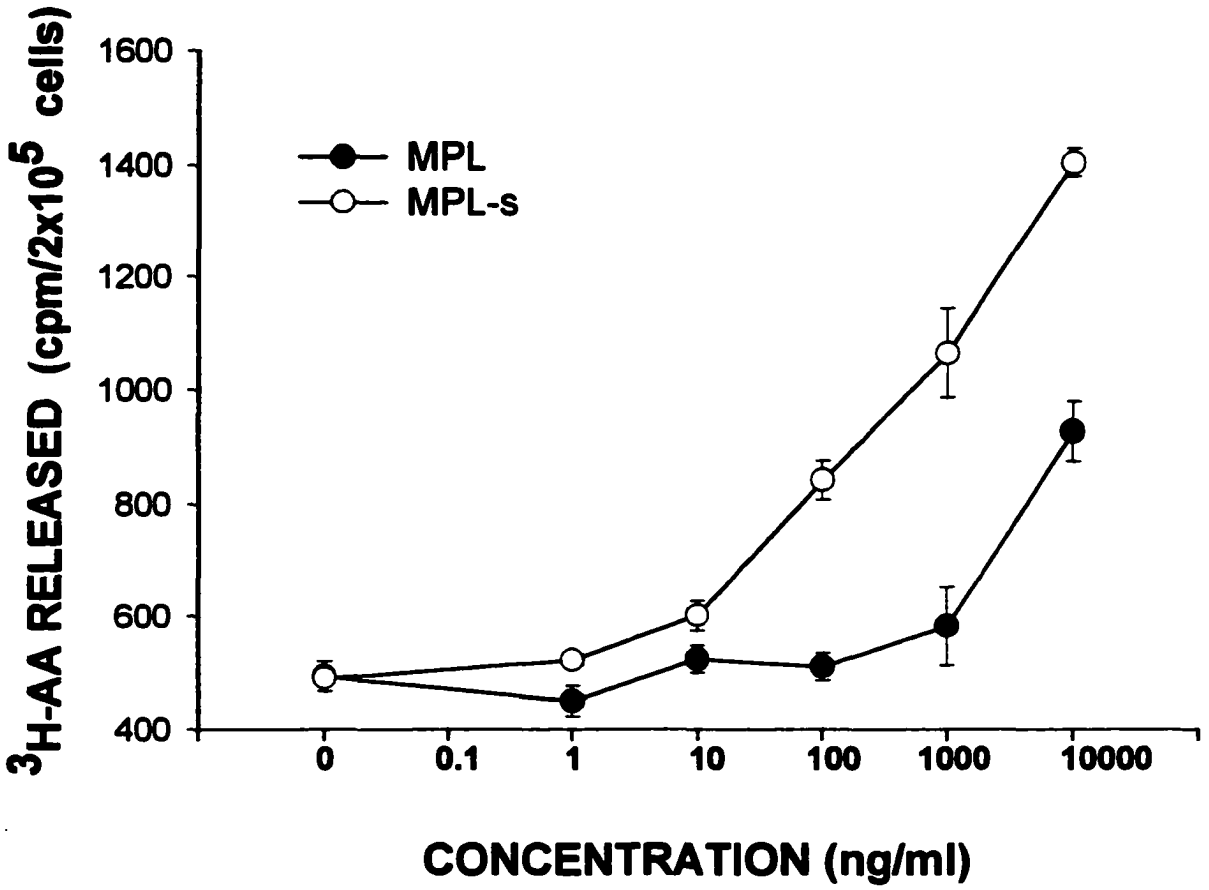


Figure 4. Difference in potency due to solute: MPL vs MPL-s. Differentiated THP-1 cells were treated as in Fig. 3, and challenged with either MPL or MPL-s for 2 hours. 200 µl of cell-free supernatants from each sample were counted for cpm released. Values are averages of duplicates, ± range.

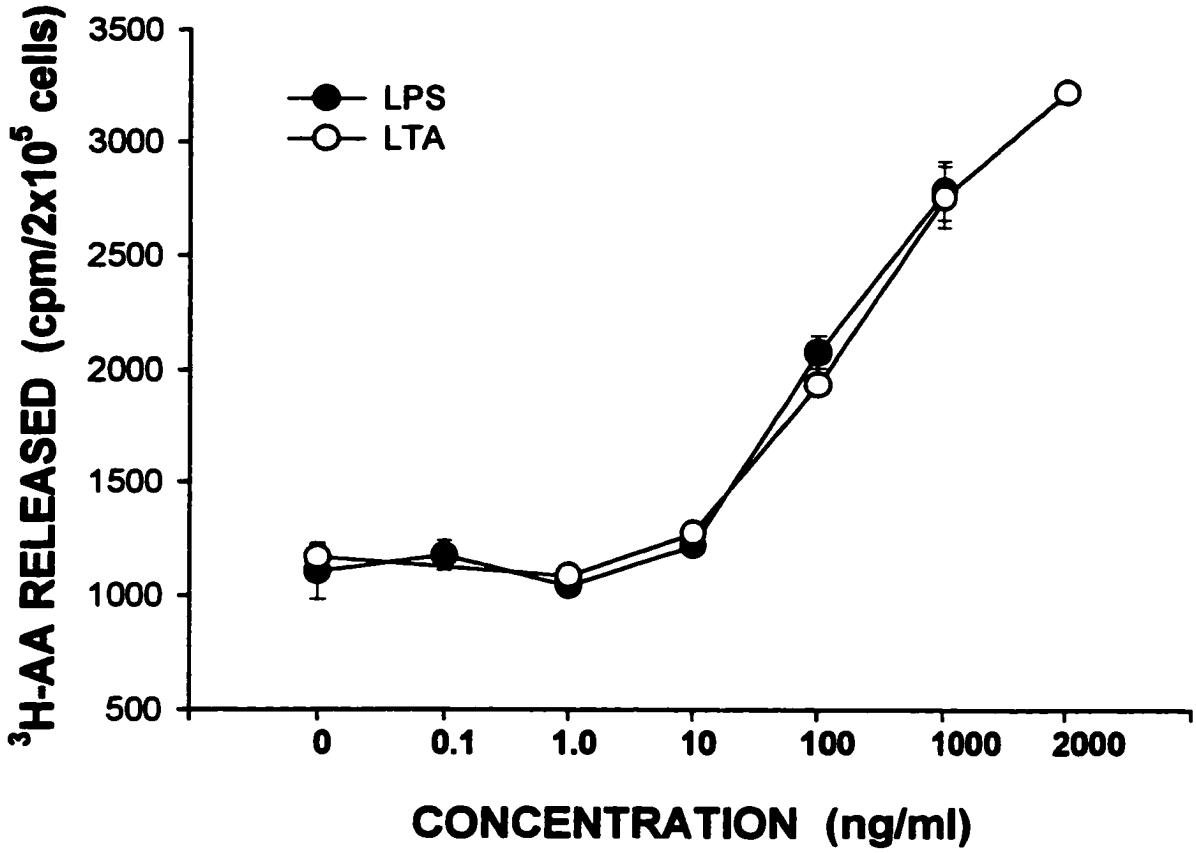


Figure 5. Effect of LPS and LTA on arachidonic acid released from RAW264.7 murine macrophages. RAW264.7 cells were plated in 24-well plates at 1x10^6 cells/ml and allowed to adhere. They were labeled overnight with ^{3}H-AA, and then gently washed with warm RPMI. The cells were challenged with LPS R595 or LTA (*E. faecalis*) for 2 hr. 200 µl of supernatants were counted for cpm released. Values are the averages of duplicates ± range.

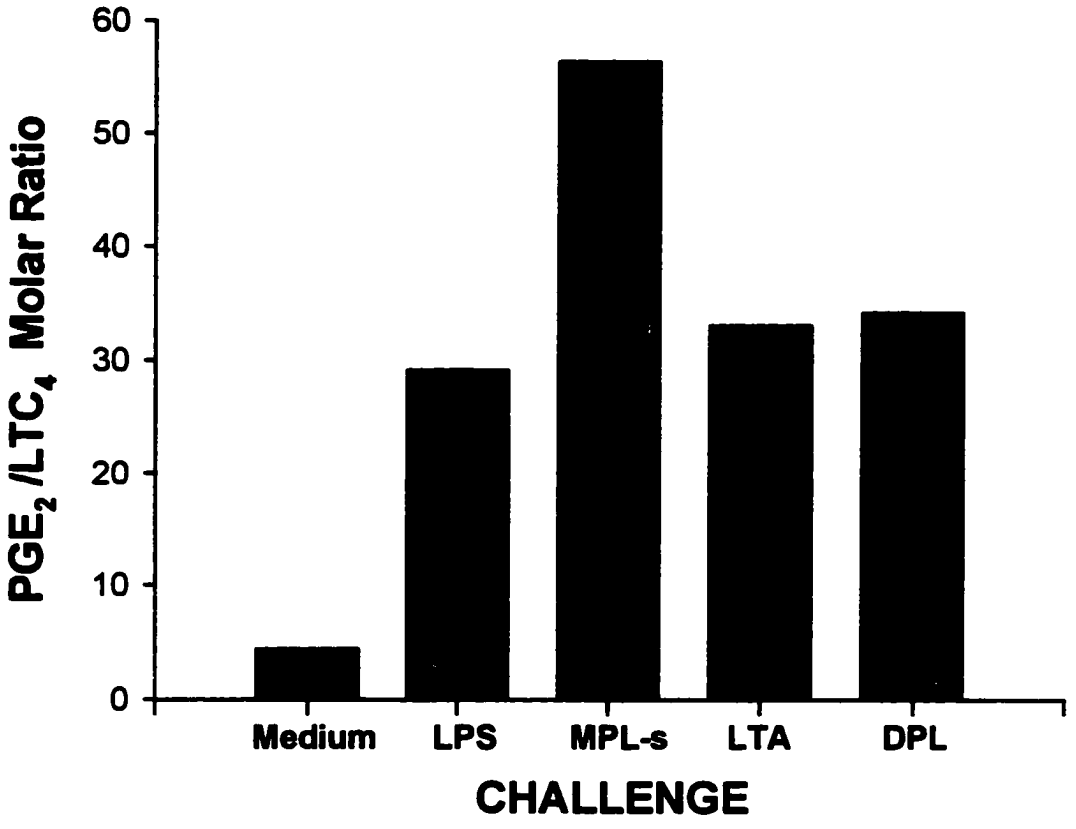


Figure 6. PGE_2/LTC_4 ratios measured from THP-1 cells challenged with LPS, MLA, DPL, or LTA. Differentiated THP-1 cells were plated in 24-well plates at $5x10^5$ cells/well in RPMI with 5% FBS, and challenged in 1 ml/well with LPS R595, MLA, DPL, or LTA (*E faecalis*) at 1000 ng/ml. Supernatants were assayed for specific eicosanoids by EIA as per manufacturer's instructions, and converted to pg/ml from a standard curve. Molar ratios were calculated for PGE_2/LTC_4. Values are the averages of duplicates.

Desensitization of macrophages by bacterial amphiphiles. MLA has been shown to protect mice and humans from the lethal effects of LPS (3,40). Our data show that a 18 hr pretreatment with MLA followed by LPS R595 challenge reduced TNFα release from THP-1 cells by up to 92% (Fig. 7).

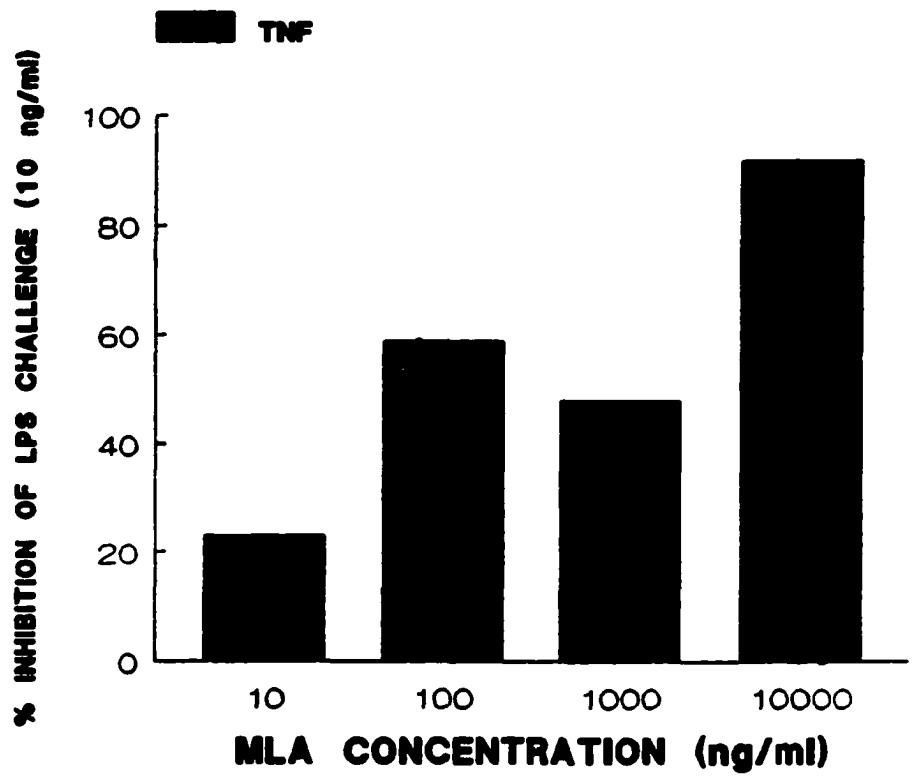


Figure 7. Desensitization by MLA to LPS challenge: TNFα release. Differentiated THP-1 cells were pretreated with 100 ng/ml MLA for 18 hours and then challenged with 10 ng/ml LPS R595 for 6 hours. Supernatants were collected stored at –70°C until assayed by ELISA for TNFα, using Quantikine Kits following manufacturer's instructions. The % inhibition was calculated based on the amount of TNF released by 10 ng/ml LPS without pretreatment with MLA.

A 2-3 hour pretreatment with MLA followed by washing and labeling of the cells, reduced eicosanoid release over 50%, even 18 hours after removal of the MLA (Fig. 8).

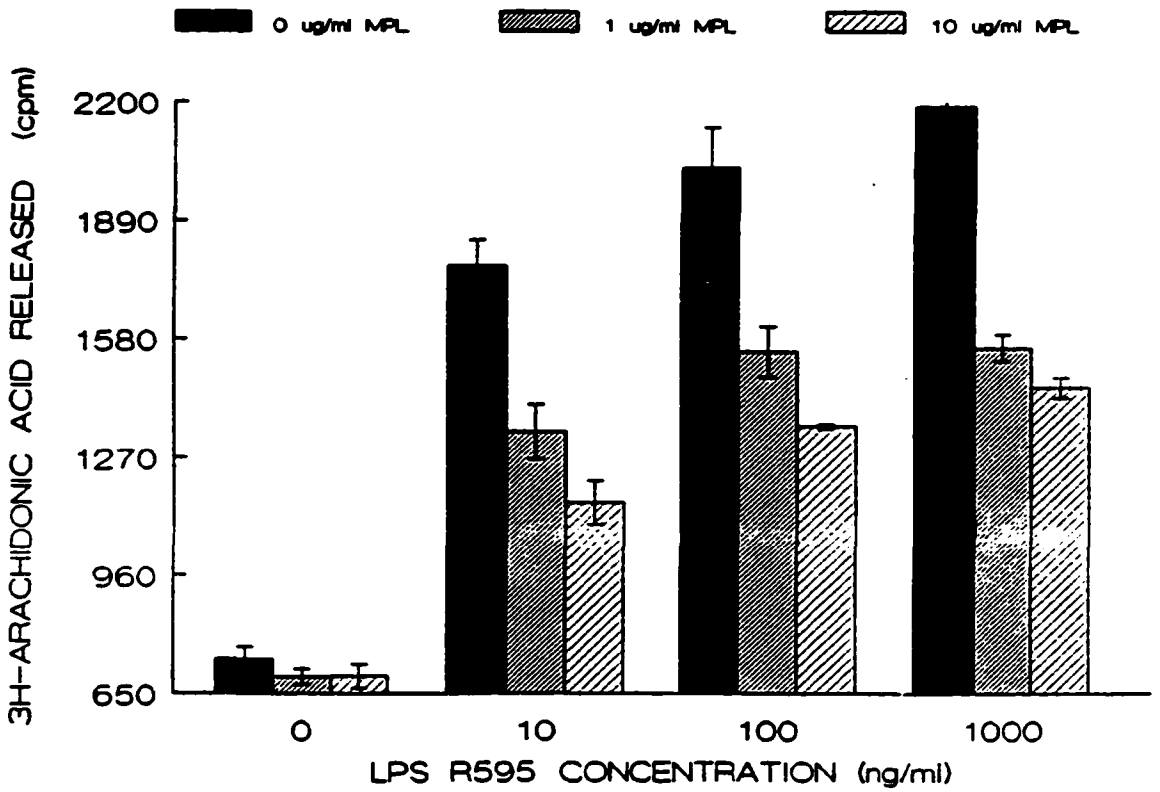


Figure 8. Desensitization by MLA to LPS challenge: ^{3}H-AA release. Differentiated THP-1 cells were pretreated with MLA at 0, 1, and 10 μg/ml for 2 hours, then washed and labeled overnight as in Fig. 1. Cells were washed 3x and challenged for 3 hours with LPS R595 (1 μg/ml). 200 μl of supernatant samples were collected and counted for cpm released. Values are averages of duplicates.

Pretreatment for 2-3 hours with LTA also desensitized cells to subsequent challenge with LPS. This was shown in RAW264.7 cells (Fig. 9), and also in THP-1 and mouse PECs (data not shown).

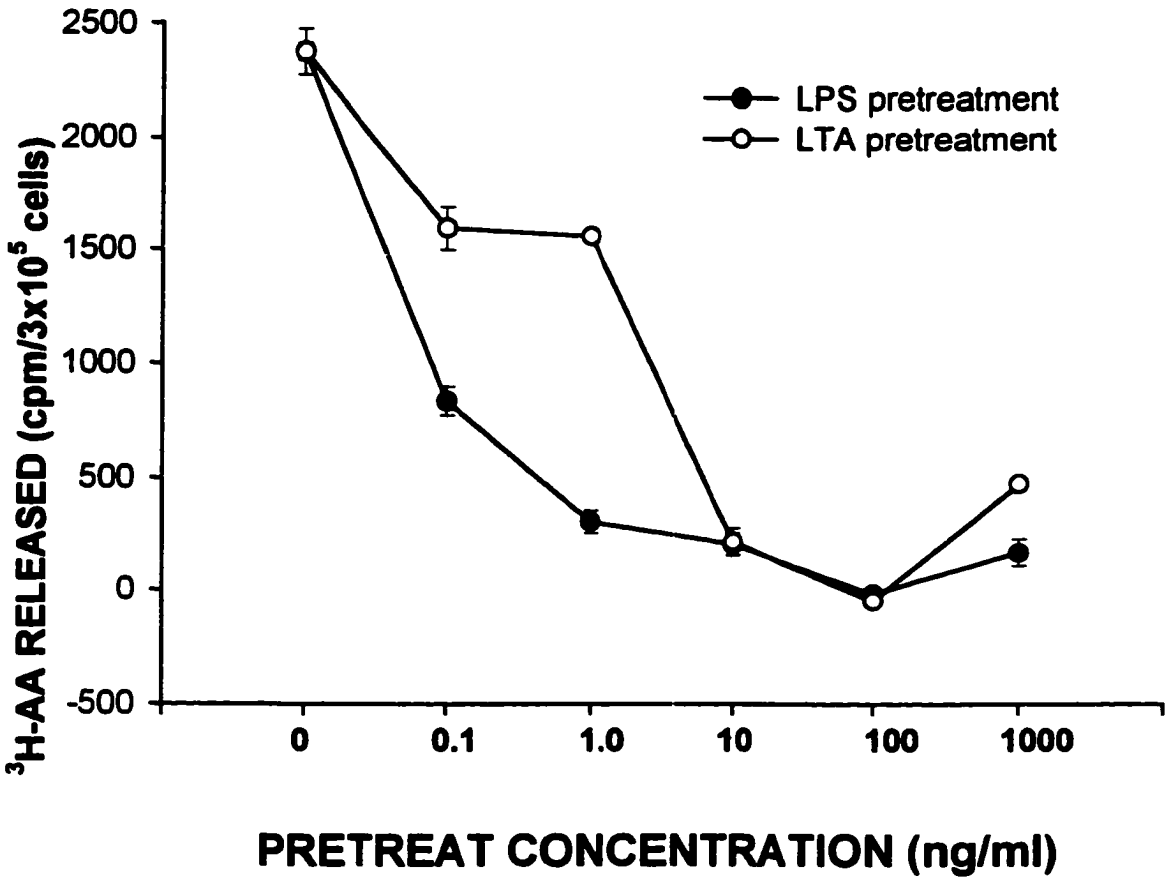


Figure 9. Desensitization of RAW cells by LTA to LPS challenge: ^{3}H-AA release. RAW264.7 mouse macrophages were plated at 1x10^6 cells/ml in 24-well plates and allowed to adhere for 2 hr. They were pretreated with LPS (R595) or LTA (*E. faecalis*) for 2 hours at concentrations shown, then washed and labeled overnight as in Fig. 1. The cells were then washed and challenged with LPS R595 at 1 μg/ml for 2 hours. Values cpm above control and are averages of duplicates, ± range.

Challenge of THP-1 cells with a vortexed mixture of MPL and LPS R595 at a 10:1 mass ratio resulted in a dramatic attenuation of the release of eicosanoids seen with LPS alone (Fig. 10).

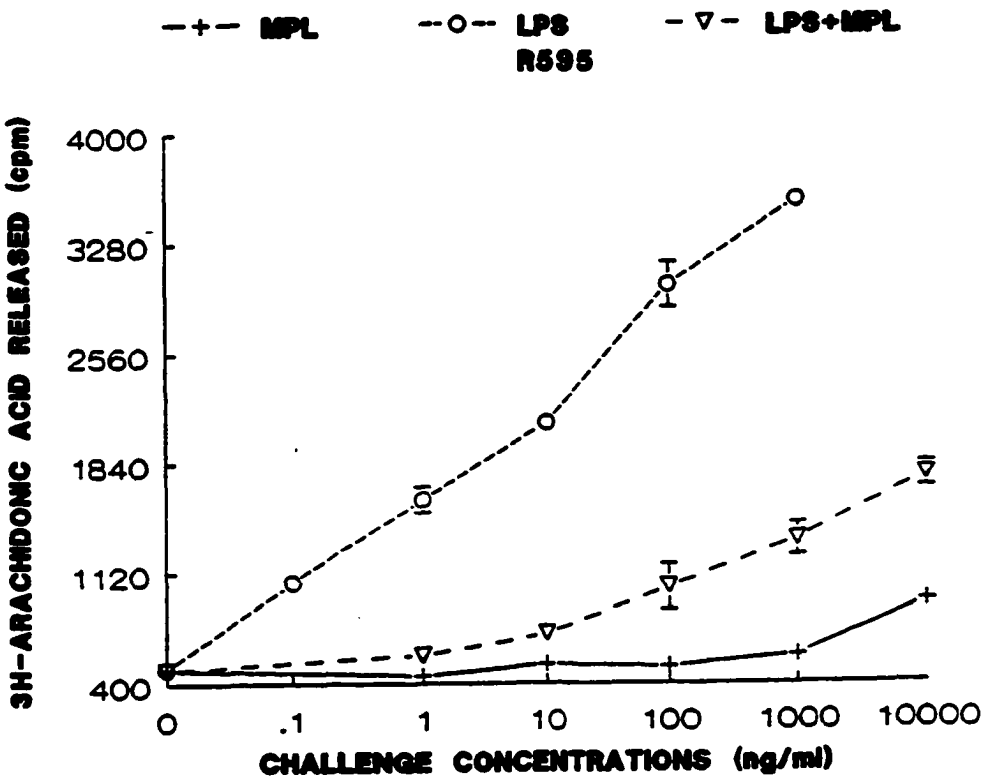


Figure 10. Effect of LPS/MPL mixed challenge on ^{3}H-AA release. THP-1 cells were labeled as in Fig. 1 and challenged with LPS R595 and MPL for 2 hr. Mixed challenge consisted of dilutions of MPL and LPS vortexed together in a 10:1 mass ratio (MPL:LPS). When graphing the mixed challenge, the MPL concentrations were used to plot points. Values represent the amount of label in 200 µl of supernatant samples counted for cpm released, and are averages of duplicates, ± range.

However, if MPL was added 1 hour prior to the addition of LPS (10:1 mass ratio), the attenuation of LPS potency was not seen (Fig. 11).

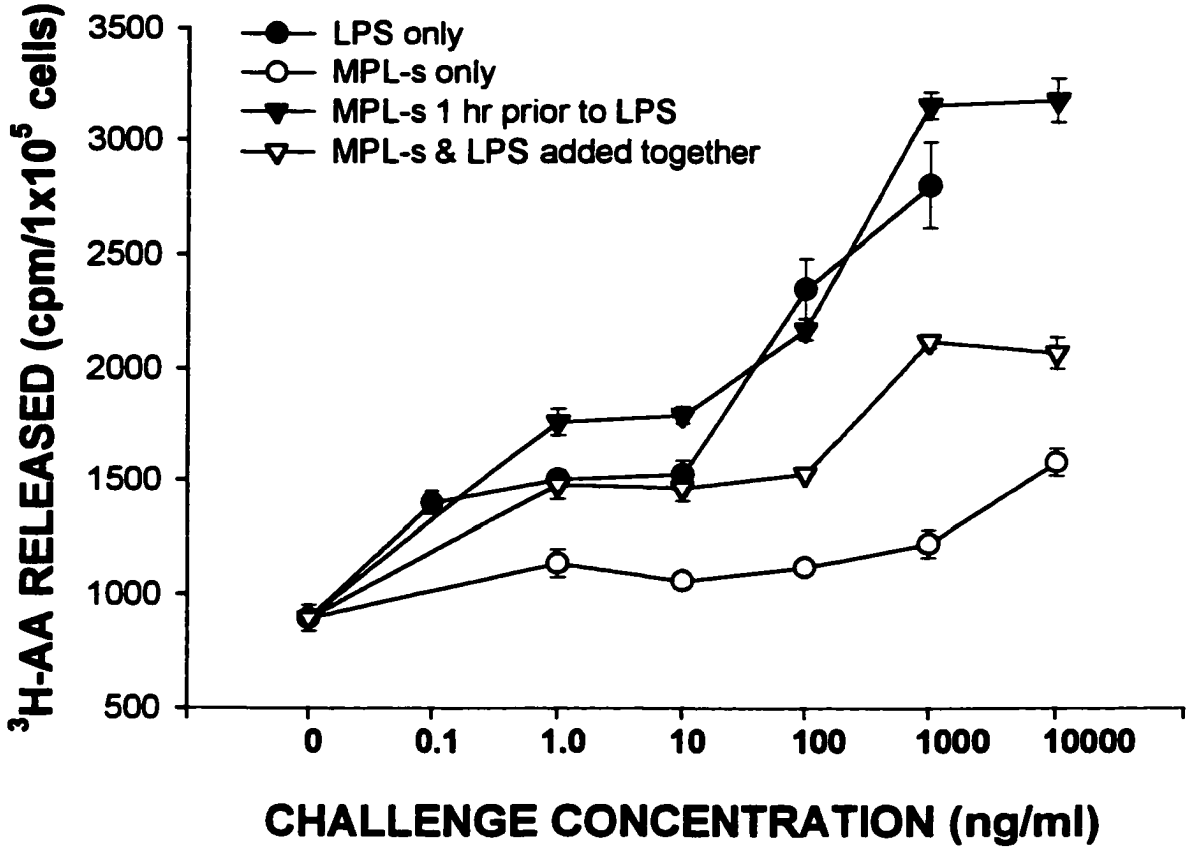


Figure 11. MPL/LPS mixed challenge, adding MPL prior to LPS to allow binding time. THP-1 cells were challenged as in Fig. 10. However, for one set of data, the MPL and LPS were not vortexed together. Instead, the MPL was added 1 hr prior to addition of LPS. Again, for mixed challenges, MPL concentrations are shown. Values are averages of duplicate 200 µl samples of cell-free supernatants, counted for cpm released.

DISCUSSION

It is widely accepted that the biologically active constituent of lipopolysaccharide is lipid A, and it is strongly implicated as the cause of endotoxic shock. However, studies have shown that the potency of LPS molecules can be modulated by the configuration of the polysaccharide portion (35,65) or the acyl chains (14). Lipoteichoic acids appear to be at least partially responsible for a similar condition called Gram positive septic shock, and they also show variations in potency when derived from different Gram positive organisms (10,16). The purpose of this study was to quantify lipid mediators released from macrophages challenged or desensitized with different LPS and LTA preparations. These data would provide a basis for developing hypotheses regarding the mechanisms behind the variable potencies, and the desensitization phenomenon. The results reported here are some of the few comparative studies of eicosanoids released from human THP-1 cells following challenge with bacterial amphiphiles.

The relative potencies of the various amphiphiles were LPS R595 ≥ DPL > *S. abortus* LPS and *E. faecalis* LTA >MLA. It is not clear what events occur at the cell surface to attenuate the signal transduction events. LPS, LTA (19,45,59), and MLA (unpublished data) have all been shown to bind the putative receptor, CD14. CD14 has been described as a "pattern recognition" receptor (75), meaning that the binding requires a particular spatial arrangement of charges. It also has been shown to require monomeric binding so that aggregates such as micelles are quite inactive (85). With this information, it is possible to visualize several reasons for the variations in activity of the amphiphiles that bind CD14. First, the stability of the micelle will affect the rate at which conversion

to monomers occurs. In aqueous solution, less stable aggregates will produce a higher concentration of monomers available for binding to CD14, and these molecules would most likely show higher potency simply due to availability. Structural variations of the fatty acids (length, fluidity), the hydrophilic core cross-sectional area, and the charge density of the polar side groups will all affect micelle stability and overall supramolecular configurations. Lipopolysaccharide binding protein (LBP) has been shown to bind LPS monomers and facilitate binding to CD14. This dramatically increases cell responsiveness to LPS. LTA has also been shown to be much more potent in the presence of serum, suggesting that a serum protein may play a similar role for delivery of these amphiphiles (16). Therefore, structural features of the particular amphiphile in the micelle may also affect the ability of LBP (or other serum protein) to bind and disperse the micelle for delivery to CD14.

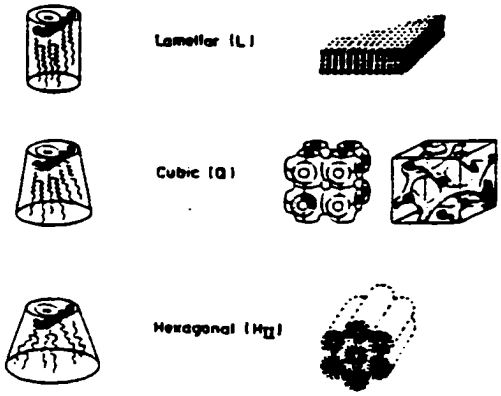
Second, slight alterations in the charge pattern of the ligand's binding site may strongly affect its affinity for CD14. This has been shown to be true with the binding of various LTAs to the scavenger receptor on macrophages, which is another pattern recognition receptor (38). Third, there is increasing evidence that CD14 is more of a delivery vehicle than a signal transducer, permitting internalization of the ligand via vesicle formation (97). Activation would then occur after binding to another receptor. Deacylated, biologically inactive lipid A derivatives have been shown to compete for binding sites on LBP and CD14, and block LPS responsiveness (56,57). This suggests that even inactive forms of LPS bind CD14, but do not transduce the activation signal. Structural variations would therefore affect binding to subsequent receptors so that

various amphiphiles might simply be less active due to lower affinity, might serve as an antagonist, or might activate different pathways resulting in different patterns of mediators released.

The data reported here combined with studies of the supramolecular structure of various amphiphiles support the micelle stability model of potency. All of these amphiphiles were capable of desensitizing the cells to subsequent challenge. This suggests common pathways involved in that regulatory mechanism. Also, all of the amphiphiles tested activated similar ratios of PGE_2 /LTC_4, suggesting that the same pathways were activated. In another study with mouse macrophages, smooth and rough LPS, LTA, and MLA all caused the release of similar PGE_2/LTC_4 ratios of approximately 10:1 (16). Interestingly, Luderitz, et al. (65) showed that lipopolysaccharides of rough mutant enterobacteria induced both LTC_4 and PGE_2 from mouse peritoneal macrophages, whereas wildtype (smooth) LPS preparations caused the release of PGE_2 but not LTC_4. The reasons for these different results are unknown.

Correlations between monomer structures and potencies have not been found for LPS or LTA (10,78). However, Brandenburg, et al. (14) showed that variations in acyl and saccharide structure combine to produce unique three-dimensional aggregates of the lipid A derivatives in aqueous environments, and that the ability of the molecules in a sample to adopt non-lamellar inverted structures is closely correlated with endotoxic potency (Fig. 12). Inactive forms of LPS, however, form lamellar structures including micelles. However, since several different factors affect aggregate formation, it is unlikely that potency is related to any single monomeric structure. More likely, a set of

conditions yield a configuration that forms a particular type of aggregate. It is not known whether the toxicity is due to the aggregation itself or to the configuration that yields the aggregate above a certain concentration. It is possible that the critical factor is the stability of the aggregate and therefore the availability of monomers for binding to target receptors.



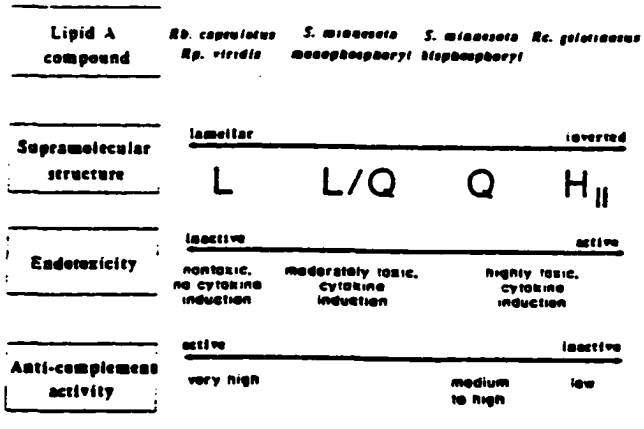


Figure 12. TOP: The relationship between molecular conformation and subsequent supramolecular structures. From Seydel, et al., 1993 (82). Phase behavior, supramolecular structure, and molecular conformation of lipopolysaccharide.
BOTTOM: The relationship between supramolecular structure and toxicity. From Brandenburg, et al., 1993(14). Influence of the supramolecular structure of free lipid A on its biological activity.

LTA from *E. faecalis* is of the Type I structure, consisting of an unbranched 1,3-linked polyglycerophosphate chain linked to a membrane diacylglyceroglycolipid containing one (20) or two fatty acid tails (38) (Figure 13). This type lacks compensatory charges for the heavily negatively charged polyglyceroposphate residues, and includes some of the more active LTAs (eg. LTA from *E. faecalis, S. aureus*) (16). Since the highly charged outer rim of the Type I LTA micelle would tend to make the micelle less stable, this suggests that micellar stability could be a factor in LTA potency as well. The critical micellar concentration (CMC) for LTA has been reported as 1-10 μg/ml (103) and 28-60 μg/ml (20). Despite this range of reported data, LTA clearly requires relatively high concentrations of monomers before micelles begin to form, possibly due to the size of the hydrophilic portion of the molecule. Our data show that LTA (*E. faecalis*) activates arachidonic acid metabolism well below CMC, as low as 10 ng/ml, and levels out above 10 μg/ml (unpublished data). This is consistent with the idea that as micelles form, the concentration of monomers in solution remains constant, in a dynamic equilibrium with molecules in aggregate form.

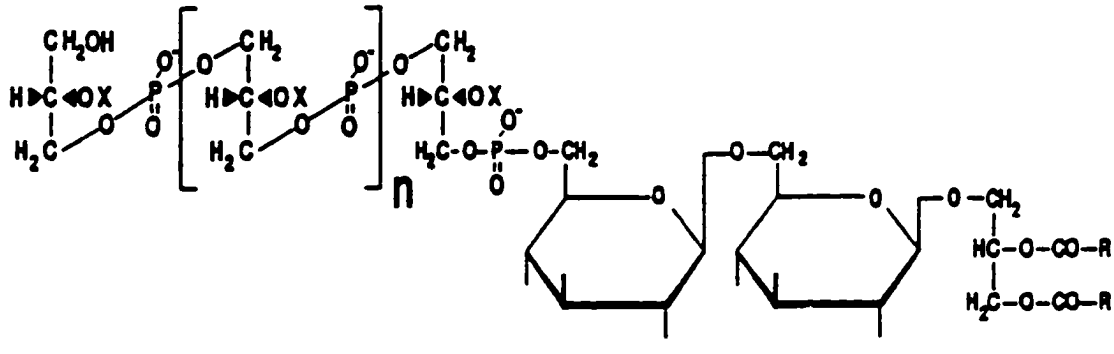


Figure 13. Type I Lipoteichoic Acid.

The micellar stability model is also supported by the fact that two preparations of MLA, differing only in their solutes, have shown significant differences in potencies for arachidonic acid release. MLA in 1% TEA (MPL) was much less active than MLA solubilized in ethanol (MPL-S) (Fig. 14). MPL-S has been shown to have better solubility than MPL. The MLA used in these experiments was prepared from R595 LPS and lacks the phosphate group and the 3' acyl chain on the reducing glucosamine.

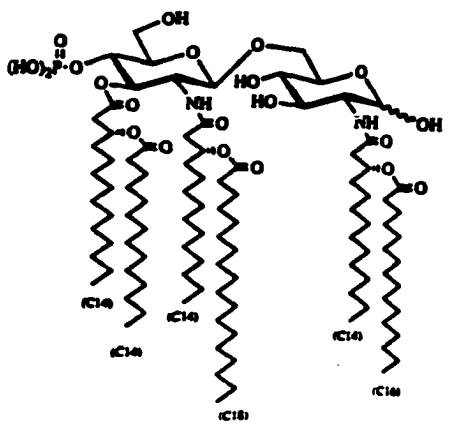


Figure 14. Monophosphoryl Lipid A (MLA, MPL)

MLA has been shown to form cubic or lamellar structures due to the lower number of fatty acids and the reduced charge density of the glucosamine backbone (82). This type of aggregate is correlated with reduced biological activity (14) and might explain some of the reduced potency of this lipid A preparation for arachidonic acid release. R595 LPS is prepared from the deep rough mutant of *Salmonella minnesota* R595 in which the LPS lacks both O side chain and core polysaccharide (Figure 15). R595 has a cone-shaped monomeric configuration which strongly favors non-lamellar (cubic) supramolecular aggregates (82). Its relatively high activity is consistent with reported correlations between non-lamellar structures and toxicity (14,78). The non-lamellar

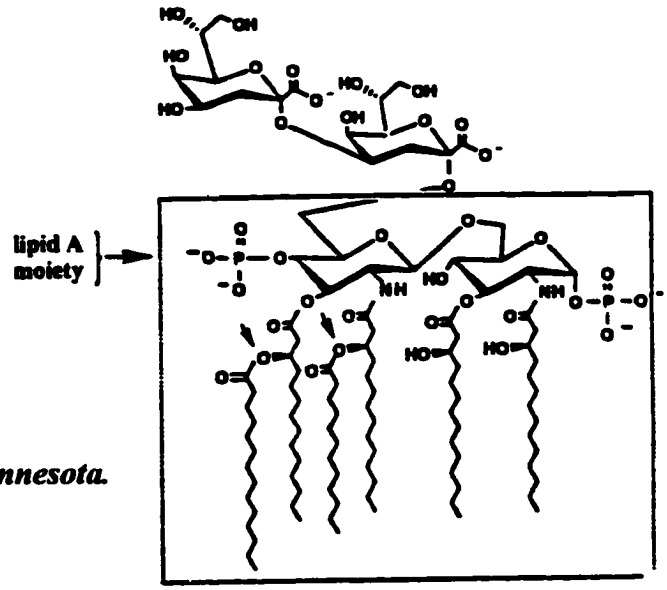


Figure 15. ReLPS from *S. minnesota.*

structures are relatively unstable in aqueous solution due to the exposure of the hydrophobic tails to the aqueous solute. The molecules involved may therefore be more potent due to the ease of transfer from the aggregate to CD14.

Diphosphoryl lipid A (DPL) is prepared by the removal of the entire inner core and KDO from R595 LPS (Figure 16). This monomer tends to adopt non-lamellar structures similar to those of ReLPS. Our data on DPL activation of arachidonic acid release (Fig. 2) support earlier work showing DPL to be 10-100 fold more lethal and pyrogenic than MLA in mouse studies (14).

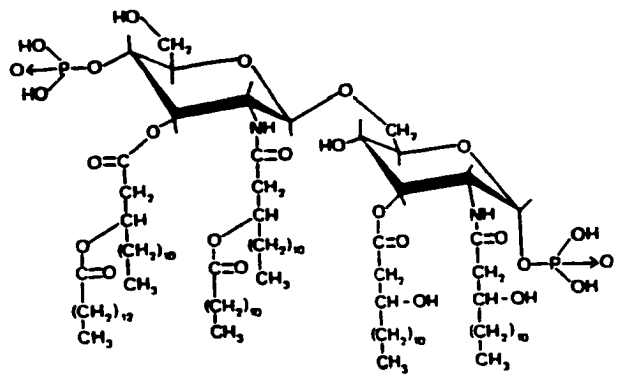


Figure 16. Diphosphoryl Lipid A (DPL).

The moderate response to LPS from *S. abortus* (Fig. 2) is interesting since its structure is of a smooth LPS, containing complete core polysaccharide and O side chain. Micelles of highly glycosylated lipids can vary considerably in stability based on the lengths of these sugar residues. The overall trend is toward more lamellar structures with increasing sugar residues (14). Therefore, our data are consistent with a more lamellar structure for smooth LPS, and therefore less activity.

When MLA and LPS were added together in a 10:1 ratio to challenge THP-1 cells, response was significantly reduced compared to challenge with LPS alone (Fig. 10). One interpretation of these results is that the MLA competed for binding and blocked activation of the cells by the more active LPS. Other studies have shown that several lipid A derivatives compete for binding at LPS binding sites (56,57). However, these same studies showed that the blockage of activation occurred at a site distinct from the binding event (56). If MLA was not actually mixed with the LPS, but was added first and allowed time to bind to the cells, the attenuating affect was not seen (Fig. 11). It seems possible that combining MLA with LPS could alter the resulting mixed aggregate from a non-lamellar aggregate to one that is more lamellar. Another possibility is that lipid A derivatives block signaling by binding to receptors by the exposed core polysaccharide while still in an aggregated form, but do not signal due to the unavailability of the acyl chains. Rietschel, et al. (79) have shown that the core polysaccharide is critical for binding, whereas the acyl chains activate signaling. However, we have shown that prebinding of MLA followed by LPS challenge did not inhibit the activation by LPS. This argues against a competitive binding type of inhibition (Fig.9).

Our results show that THP-1 cells treated with MLA, which has low toxicity, released significantly lower amounts of all mediators measured, including labeled arachidonic acid (Fig. 1), TNFα, Il-1, and IL-6 (Fig. 3). The only product shown to increase following treatment with MLA was prostaglandin, which also increased following activation with LPS or LTA (Fig. 6). Reduction of pro-inflammatory cytokines with concomitant increase in PGE_2 may be part of the reason MLA retains immunostimulatory properties and the ability to desensitize cells, yet is not endotoxic. Prostaglandin E_2 has been implicated in autocrine down-regulation of nitric oxide synthase and tumoricidal activity in mouse macrophages (76), and of TNF in a human monocytic cell line (41). It is possible that it is the release of prostaglandin that allows these agents to desensitize the cells to subsequent challenge.

This limited study on potencies of bacterial amphiphiles is not conclusive regarding a predictable model based on monomeric and/or supramolecular structures. However, it provides some evidence supporting the hypothesis that aggregate stability may play a role in the variable potencies for the release of arachidonic acid metabolites and pro-inflammatory cytokines, and therefore *in vivo* toxicity. These studies also suggest that all of these amphiphiles share similar pathways in terms of the release of specific eicosanoids, and in the phenomenon of desensitization.

CHAPTER 4

A Comparison of the Effects of LPS and Ceramide on Arachidonic Acid Metabolism in THP-1 Monocytic Cells

Introduction

Recent advances in the study of sphingolipids as mediators of signal transduction have provided new clues to the complexities of macrophage activation. In 1989, Okazaki showed that vitamin D3 treatment of HL-60 myeloid leukemia cells caused the hydrolysis of sphingomyelin, resulting in the release of ceramide with subsequent differentiation of the cells and growth arrest (73). Since then, many studies have explored the role of ceramide as a second messenger in different cell types. Ceramide released through the neutral sphingomyelinase cycle (Figure 1) has been shown to transduce signaling in immune cells activated with tumor necrosis factor (TNFα) and interleukin 1 (6,54).

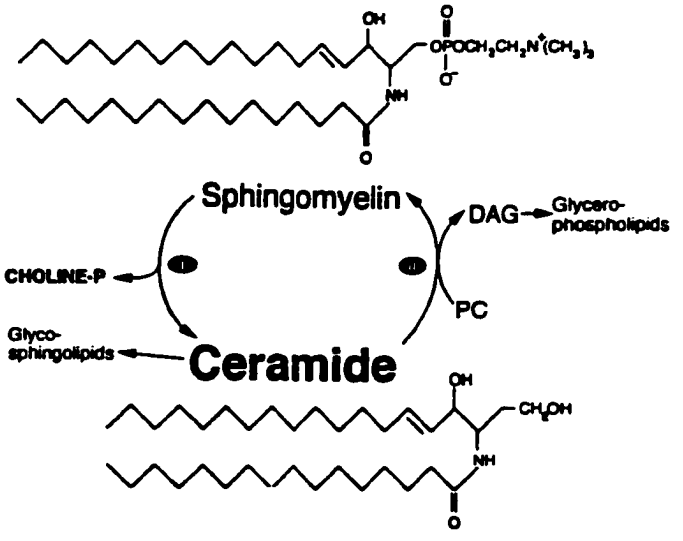
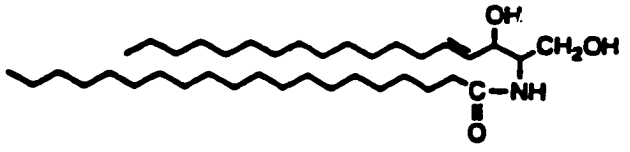
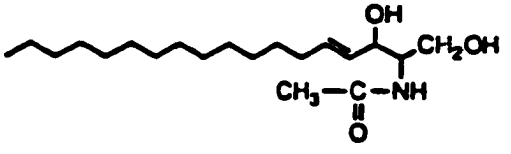


Figure 1. The Sphingomyelinase Cycle. Enzyme I = Sphingomyelinase. Enzyme II = Phosphocholine transferase. (From Ballou, 1996)

Some of the biological effects of ceramide in immune cells include cell differentiation, cell cycle arrest, and in some circumstances, apoptosis. These effects are mimicked by cell-permeable ceramides such as N-acetylsphingosine (C2 ceramide, Figure 2) (11,54). There is some evidence that exogenous bacterial sphingomyelinase (SMase) may mimic ceramide signaling by releasing endogenous ceramide, but a recent study suggests that the ceramide released is from a distinct pool that is not involved in these signaling pathways (109).



CERAMIDE



C2-CERAMIDE

Figure 2. Ceramide and C2 ceramide, a short-chain, cell permeable analog.

Research has shown that the biological response of cells activated with LPS is similar to the response of cells treated with TNFα or IL-1. However, no accumulation of ceramide was observed (52). This, along with the structural similarity between ceramide and the reducing glucosamine of the lipid A moiety of LPS, led Wright and coworkers (52,107) to suggest that LPS may exert its effects through mimicry of ceramide (Fig. 3).

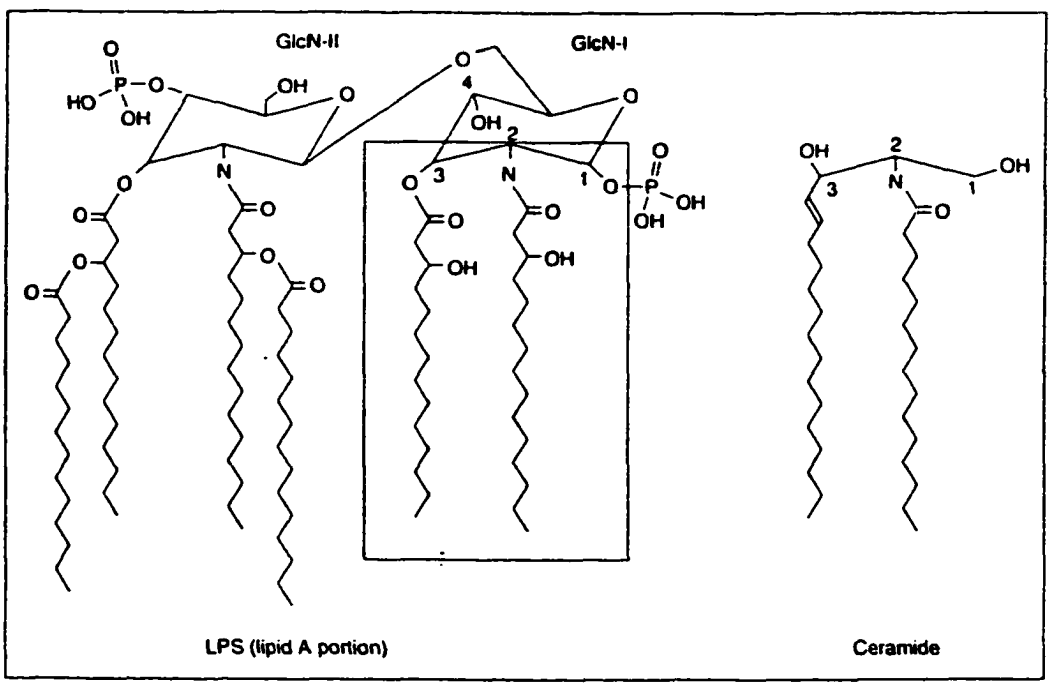


Figure 3. Structural similarity between ceramide and the acylated reducing glucosamine of Lipid A. (From Wright, 1995)

A better understanding of how LPS exerts its effects on immune cells is important due to its role as the primary mediator of septic shock. Septic, or endotoxic, shock is characterized by severe immune dysfunction and can lead to circulatory system compromise, organ failure, and death (5,13). Most of the toxicity of LPS has been traced to the lipid A portion of the molecule (79), and macrophages appear to be critical players in the immune dysfunction that leads to systemic failure (30).

Eicosanoids are biologically active arachidonic acid metabolites that are rapidly released from macrophages following activation with various agents such as LPS and phorbol myristate acetate (PMA). Free arachidonic acid is released from lipid pools by the activation of phospholipase A_2 (PLA_2), and is then metabolized to the various eicosanoids by specific enzymes. Two of the major eicosanoids released from macrophages are prostaglandins (cyclo-oxygenase pathway) and peptidyl-leukotrienes (lipoxygenase pathway) (31,33,48,105). The ratio of these two lipid families appears to be important in the regulation of the immune response (48,100,105). If LPS mimics the second messenger function of ceramide, then exogenous ceramide should cause the release of similar combinations of eicosanoids. LPS increases both PLA_2 and cyclo-oxygenase activities, but not 5-lipoxygenase in mouse macrophages (33,76,105), and human monocytes (31). Less is known about the effects of ceramide on arachidonic acid metabolism. Hayakawa, et al. (46) showed that exogenous ceramide activated transcription of PLA_2 and cyclo-oxygenase 2 in L929 cells. In HL-60 cells, it appears that the release of arachidonic acid is part of the signal transduction pathways via the TNFα and IL-1 receptors that activate the sphingomyelinase cycle (51), and therefore

occurs prior to the release of endogenous ceramide. There is also some evidence that TNF may activate two pathways via different lipid pools, one activating PLA_2 and the other leading to ceramide generation and NF-κB translocation (63,80,104). Clearly, the signal transduction events that lead to eicosanoid production in response to LPS or the sphingomyelinase pathway are complex and their interrelationships remain to be elucidated.

LPS has been shown to desensitize macrophages to subsequent challenge with LPS (96) so that the release of eicosanoids and cytokines is suppressed. If LPS simply mimics ceramide, ceramide should show similar effects. Barber, et al. (7) have recently shown that although exogenous SMase did not desensitize murine peritoneal macrophages to subsequent challenge with LPS, LPS did desensitize the cells to challenge with exogenous SMase as measured by the release of TNFα. They suggested that LPS appears to share some signaling roles with ceramide, but that the signaling pathways must diverge to produce these results. Their work was done using exogenous sphingomyelinase from *S. aureus* under the assumption that it would release endogenous ceramide in a manner similar to activation of endogenous SMase. However, Zhang, et al. (109) recently showed that exogenous SMase is unable to mimic the effects of ceramide generated by endogenous neutral SMase or by cell-permeable ceramides. It will be very important to determine which treatments most closely mimic endogenous ceramide, before the mimicry of ceramide by LPS can be elucidated in terms of signaling pathways.

In order to address these questions regarding LPS mimicry of ceramide, we compared the effects of exogenous N-acetylsphingosine (C2 ceramide) and sphingomyelinase (*S. aureus*) with those of LPS on the release of arachidonic acid metabolites from THP-1 monocytic cells, and the ability of these agents to induce a state of tolerance to subsequent challenge.

MATERIALS AND METHODS

Materials. THP-1 cells were purchased from American Type Culture Collection (Rockville MD). Cellgro RPMI 1640 was from Mediatech (Herndon VA), and defined fetal bovine serum (FBS) was purchased from HyClone (Logan UT). Purified LPS R595 from S. *minnesota* was provided by Ribi Immunochem Research (Hamilton MT). Tritiated arachidonic acid was purchased from American Radiolabeled Chemical Co. (St. Louis MO). Vitamin D3 (1α, 25 dihydroxyvitamin D) and dihydroceramide were purchased from Calbiochem (LaJolla CA). Monoclonal antibodies for flow cytometry were purchased from Becton-Dickinson Immunocytometry Systems (San Jose CA) and Coulter Laboratories. Enzyme Immunoassay kits were obtained from Cayman Chemical Co. (Ann Arbor MI). All other chemicals were from Sigma Chemical (St. Louis MO).

Cell Culture. Cells were grown in RPMI supplemented with 10% FBS, 100 U pennicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine, and maintained between 2 – 6 x 10^5 cells/ml at 37°C with 5% CO_2. Viability remained above 97% throughout the experiments except where noted. To induce expression of CD14, the putative LPS receptor and marker for a more mature (macrophage) phenotype, cells were pretreated for

24 hours with 0.1 μM vitamin D3. Cells were then labeled with ^{3}H-arachidonic acid (specific activity = 200 Ci/mmole) at 0.25 μCi/ml overnight.

Cell Activation. Prior to challenge the cells were washed with warm RPMI, resuspended in 25 ml of RPMI, and transferred to 24-well culture plates (1 ml/well). The final serum concentration was 2.5%, and final cell densities were between 5 – 8 x 10^5 cells/ml. N-acetylsphingosine (C2 ceramide) and dihydroceramide were solubilized in ethanol, and LPS was solubilized in 0.1% triethylamine. Controls were included to rule out effects of the carriers alone. Endotoxin levels in ceramide and vitamin D3 solutions were shown to be below detectable levels by Limulus assay kit (Sigma).

At the end of challenge time periods, samples were removed from the wells, cells were removed by centrifugation, and the supernatant was analyzed for released eicosanoids. The total tritium-labeled eicosanoids released from cells was determined in a Beckman scintillation spectrometer (Model LS6500). Individual eicosanoids (PGE_2, LTC_4, and LTB_4) were determined by enzyme immunoassay (EIA) analysis per the manufacturer's instructions (Cayman Chemical).

Desensitization. Cells were treated with 100 ng/ml LPS (R595), 50 μM C2 ceramide, or 0.25 U/ml sphingomyelinase (SMase) for 3 hours and then washed and resuspended in fresh RPMI with 10% FBS prior to the overnight labeling with ^{3}H-arachidonic acid (^{3}H-AA).

Flow Cytometry. After overnight treatment with D3, ceramide (C2), or LPS, THP-1 cells were stained with FITC or phycoerythrin (PE) conjugated monoclonal antibodies to CD14 epitopes including anti-CD14/FITC (MØ-P9) from Becton-

Dickinson and anti-CD14/PE (MY-4) from Coulter Labs. PE-conjugated monoclonal antibodies to constitutively-expressed leukocyte marker, CD45, were used as a positive control stain to demonstrate consistent staining of samples. The staining period was 30 min at 4°C. Cells were washed twice with staining buffer (PBS, 0.05% sodium azide, 2% FBS) and fixed with 1% buffered paraformaldehyde. PE and FITC conjugated isotype-matched negative control antibodies to myeloma proteins were used throughout to quantitate nonspecific background binding to target cells. Forward angle (FALS) and 90° (orthogonal) light scatter gating eliminated debris and dead cells from the analysis on a Coulter elite flow cytometer. Data represents the percentage of positively stained cells for a given antibody, projected beyond the cursors set on negative control histograms to separate positively and negatively stained cells. Cursors were set so that 1-2% of all cells stained with the negative control, isotype-matched reagents were displayed as being in the positive region of the histogram.

RESULTS

Sphingomyelinase and C2 ceramide activated arachidonic acid metabolism in THP-1 cells. Arachidonic acid metabolism in THP-1 cells differentiated with 0.1 μM vitamin D3, was activated by a wide range of LPS concentrations (0.1 ng/ml – 1000 ng/ml) in medium containing 2.5% FBS (Fig. 4). Activation was seen with C2 ceramide at concentrations as low as 5 μM (Fig. 4) and sphingomyelinase as low as 0.03 units/ml (Fig. 5).

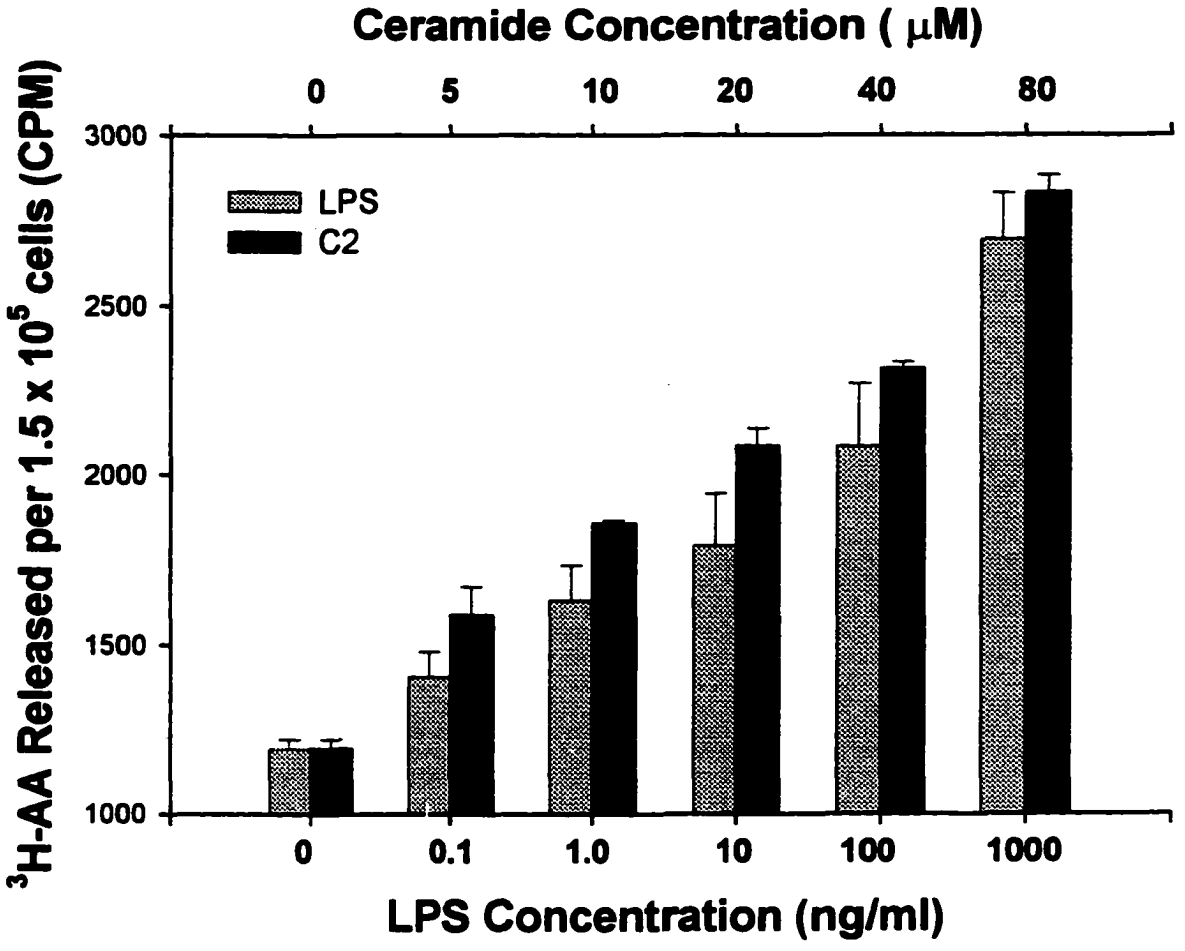


Figure 4. LPS and ceramide activate arachidonic acid metabolism in THP-1 cells. THP-1 cells were pretreated with vitamin D3 for 24 hr and labeled with 0.5 μC/ml ^{3}H-AA overnight. Cells were then washed, resuspended in RPMI with 2.5% FBS, and challenged with LPS R595 or C2 ceramide for 3 hr. Cells were removed by centrifugation, and 200 μl of supernatant were counted for cpm released. Values are the averages of duplicate samples ± range.

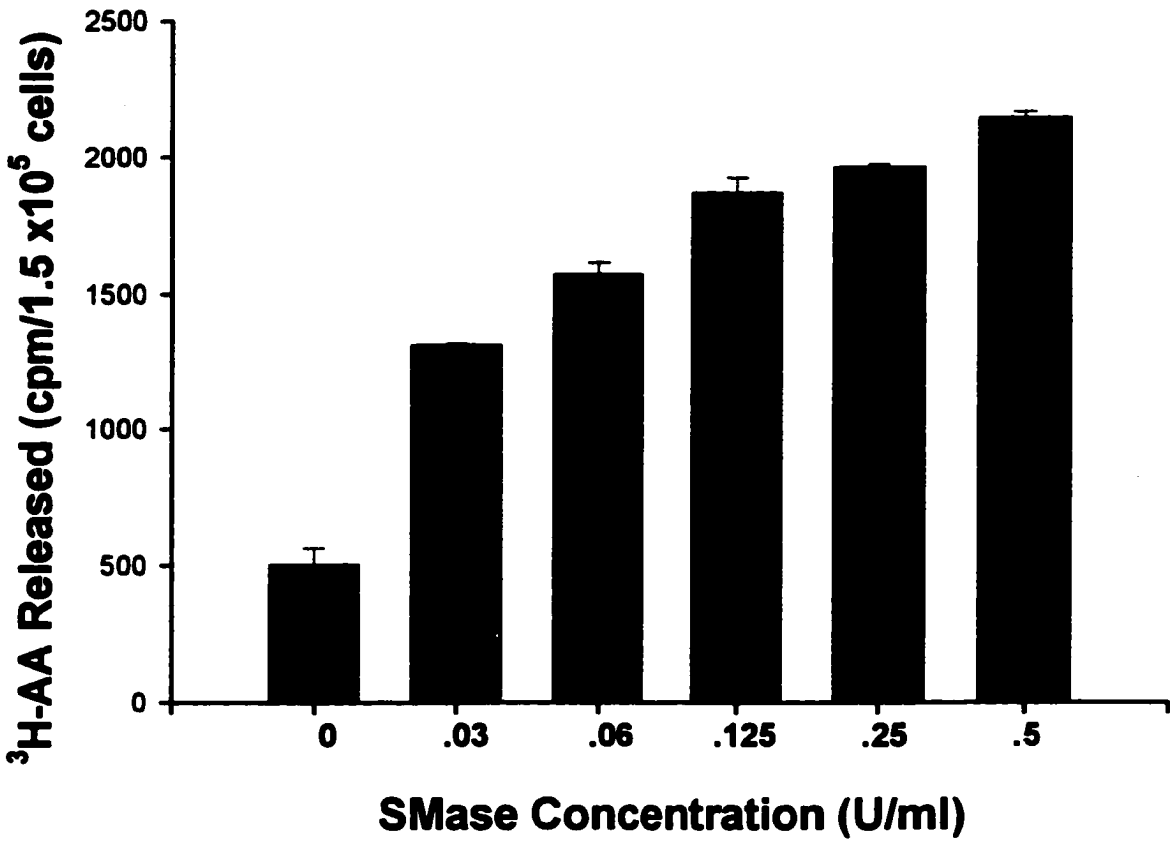


Figure 5. Activation of arachidonic acid metabolism by exogenous bacterial sphingomyelinase. Cells were treated as in Fig. 1 and then challenged with SMase for 3 hr. Cells were removed by centrifugation, and 200 µl of supernatants were counted for cpm released. Values are averages of duplicates ± range, and represent at least two experiments.

Cytotoxicity, as measured by trypan blue uptake, was observed at concentrations of LPS above 1000 ng/ml, C2 ceramide above 100 μM, and sphingomyelinase above 1 unit/ml. Dihydroceramide, due to poor uptake by cells, was used as a negative control, and elicited little or no response (data not shown).

The response of THP-1 cells to simultaneous challenge with both LPS and ceramide depended on concentrations of these agents. At lower concentrations of both LPS and ceramide, release was slightly higher than with either agent alone (Table 1), but at higher concentrations of LPS, the addition of ceramide had little effect.

TABLE I
Concomitant challenge of THP-1 cells with LPS and ceramide.[a]

	LPS Concentration (ng/ml)					
	0	.1	1	10	100	1000
Ceramide						
0 μM	0	405(16)[b]	441(5)	619(6)	1436(88)	1844(61)
40 μM	208(9)	605(11)	691(33)	764(64)	1503(48)	1789(97)
60 μM	359(5)	852(35)	840(5)	987(13)	1446(21)	1664(15)

a) THP-1 cells were treated and labeled as in Fig. 1 and challenged for 2 hr with LPS (ng/ml) and C2 ceramide (μM) alone and together. 200 μl of post-challenge supernatant were counted for label released from the cells.

b) Values show cpm released from 1.5×10^5 cells above control values, and are averages of duplicate samples ± range.

Prostaglandin E_2/Leukotriene C_4 molar ratios were higher from LPS-treated cells than from ceramide-treated cells. Untreated (control) cells released 40 ±9 pg/ml of PGE_2 and 23 ±11 pg/ml of LTC_4 during the 3 hour incubation period. The ratio of PGE_2/LTC_4 in the lipid released from LPS treated cells was about 10 times the value for untreated cells. In contrast the molar ratio of PGE_2/LTC_4 in the lipid released from ceramide treated cells remained constant (Fig. 6a). This difference resulted from a higher production of prostaglandin from LPS treated cells (Fig. 6b). Interestingly, the PGE_2/LTC_4 ratio increased as the concentration of LPS increased, but remained fairly constant at all concentrations of ceramide even though there were increases in both PGE_2 and LTC_4 to approximately 100 pg/ml. This suggests that whereas LPS activated cyclo-oxygenase activity above constitutive levels, ceramide increased PG and LT release only through increased availability of arachidonic acid via PLA_2 activation, and not through specific induction of cyclo-oxygenase or lipoxygenase pathways. The amount of PGE_2 from cells treated with SMase was very similar to that of LPS-treated cells, increasing to give a PGE_2LTC_4 ratio that was a log higher at maximal SMase concentration than constitutive levels from untreated cells (Fig. 6c). LTB_4 did not significantly increase following challenge with any of the activators (data not shown).

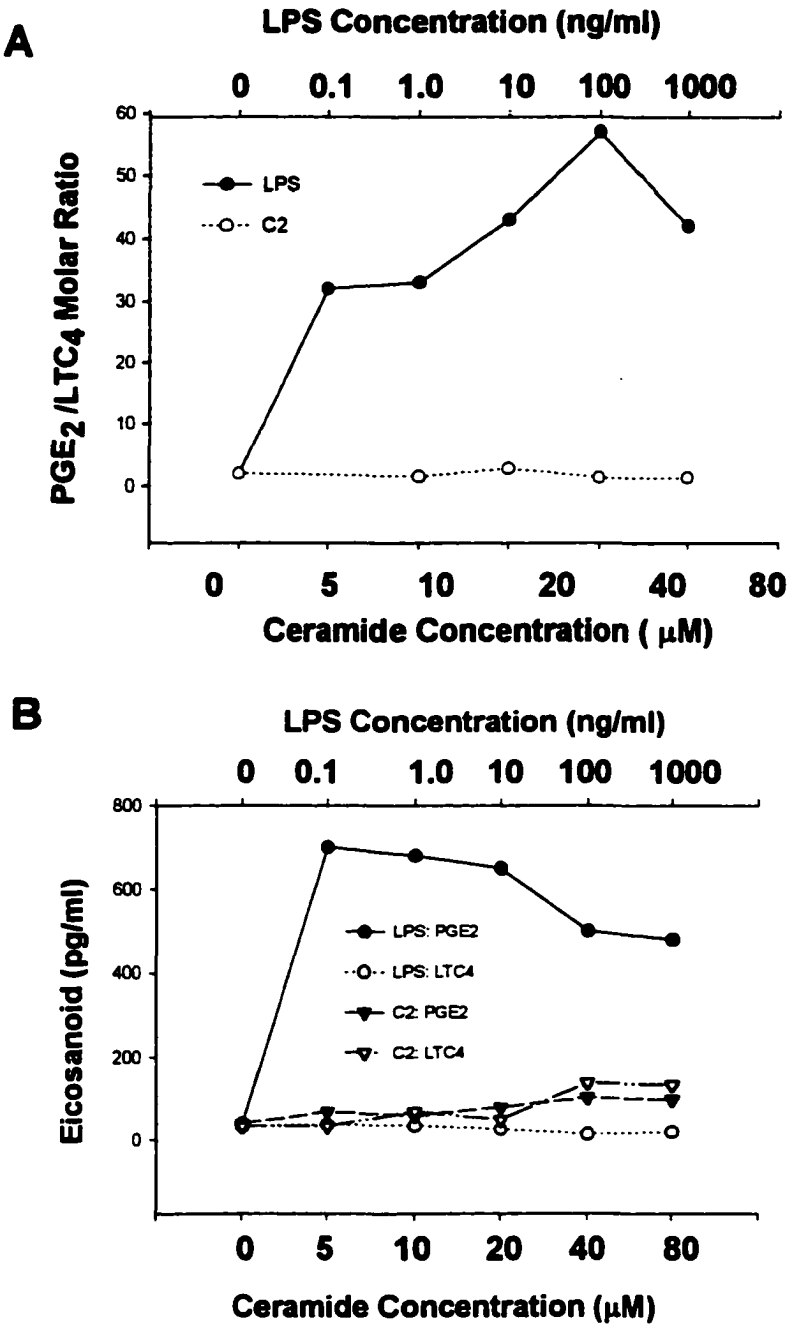


Figure 6 A & B. PGE_2/LTC_4 molar ratios released from THP-1 cells. Supernatant samples from experiments performed as in Fig. 1 and 2 were assayed for PGE_2 and LTC_4 by EIA, following procedure provided with the assay kits. A) Ratios in pg/ml were converted to molar ratios and plotted for cells treated with LPS (top axis) and C2 ceramide (bottom axis). B) Quantities of PGE_2 and LTC_4 from A expressed as pg/ml. Values are averages of duplicate samples.

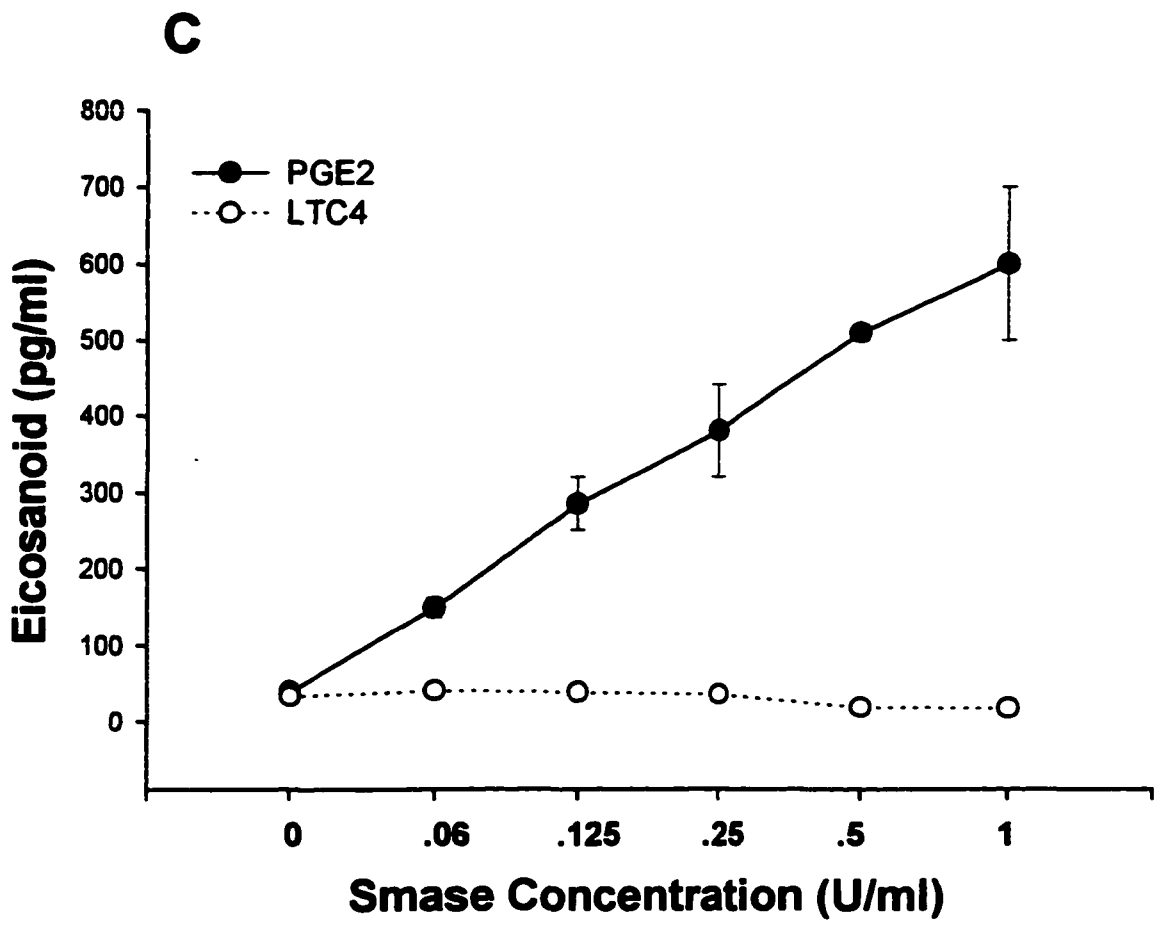


Figure 6. (continued) C) PGE_2 and LTC_4 in pg/ml from cells treated with SMase. Values are the averages of duplicates.

Desensitization of THP-1 cells. Pre-exposure to LPS induces a tolerant state during which subsequent challenge with LPS fails to activate several pathways (27,41,96). In our studies, an initial 2-3 hour treatment of THP-1 cells (or murine peritoneal macrophages) with 100 ng/ml LPS desensitized them to subsequent challenge with a wide range of LPS concentrations so that eicosanoid release remained near control levels even 20 hours after pretreatment. To determine whether ceamide had the same effect, we treated THP-1 cells with either LPS (100 ng/ml), ceramide (50 μM), or SMase (0.25 U/ml) for 3 hours, then labeled the cells overnight with ^{3}H-AA. Cells were then challenged with LPS, ceramide, or SMase (Fig. 7). In terms of ^{3}H-AA release, LPS desensitized cells to challenge with LPS (Fig. 7A), and partially to challenge with SMase (Fig. 7C). Pretreatment with SMase desensitized cells to challenge with SMase (Fig. 7C) and partially to challenge with LPS (Fig. 7A), but primed them for challenge with C2 ceramide (Fig. 7B). Pretreatment with C2 had very little effect on LPS or C2 challenge (Figs. 7A&B), but primed the cells for SMase challenge (Fig. 7C).

In order to determine whether pretreatment affected the PGE_2/LTC_4 ratio in the lipid released from cells after subsequent challenge, the amounts of PGE_2 and LTC_4 were determined for supernatants of cells pretreated with either medium alone (control), LPS (100 ng/ml), C2 ceramide (40 μM), or Smase (0.25 U/ml), then challenged with 100 ng/ml LPS. Pretreatment with LPS almost completely blocked the increase in PGE_2 production following subsequent challenge with LPS. Interestingly, both SMase and C2 ceramide also reduced the amount of PGE_2 produced after LPS challenge, but only to about half the amount produced from non-pretreated cells (Fig. 8).

Figure 7. Pre-exposure to LPS, but not ceramide, desensitized THP-1 cells to subsequent challenge. THP-1 cells were pretreated with D3 as in Fig.1, then treated with medium alone (control) (•), 100 ng/ml LPS (o), 0.25 U/ml Smase (Δ), or 50 μM C2 ceramide (▲) for 3 hr. Cells were resuspended in fresh medium and labeled overnight with ^{3}H-AA. They were then washed and challenged with LPS (A), ceramide (B), or SMase (C) for 3 hr. Values show relative response of cells after various treatments as the amount of label released per $1.5x10^{5}$ cells divided by the amount of label released from unchallenged control for each sample, and are averages of duplicates.

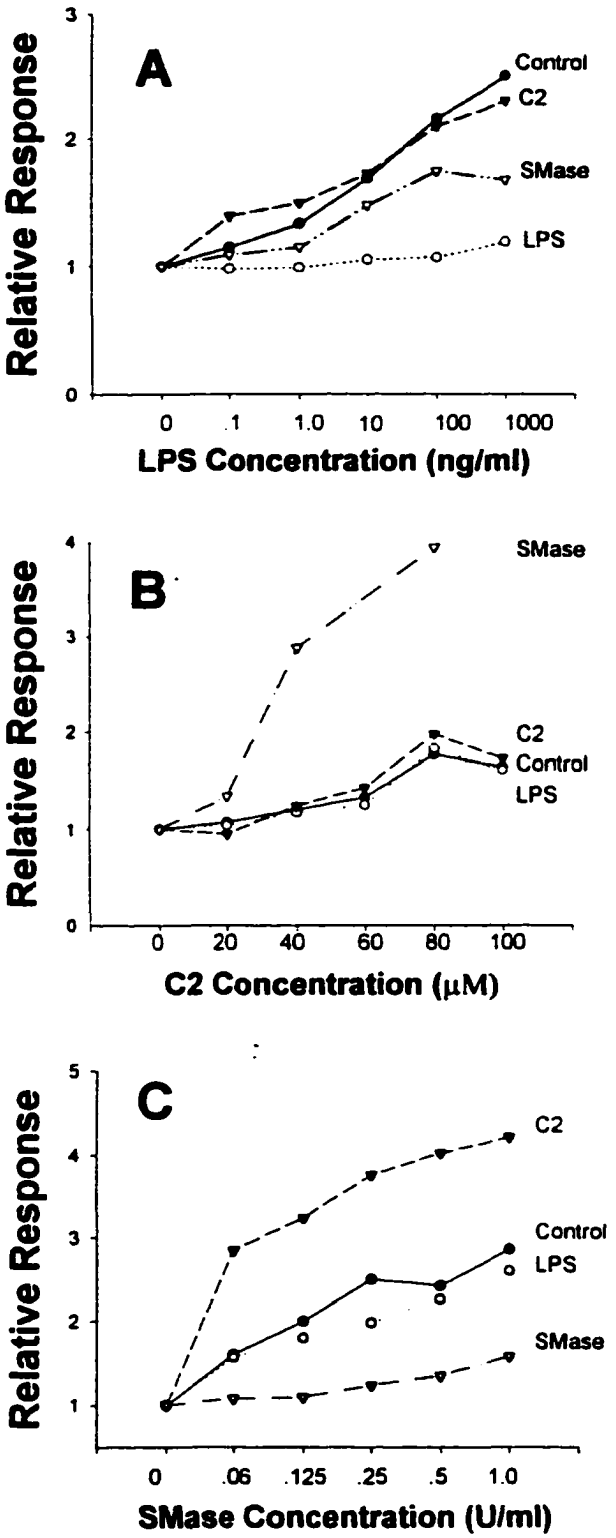


Figure 7. Pre-exposure to LPS, but not ceramide, desensitized THP-1 cells..
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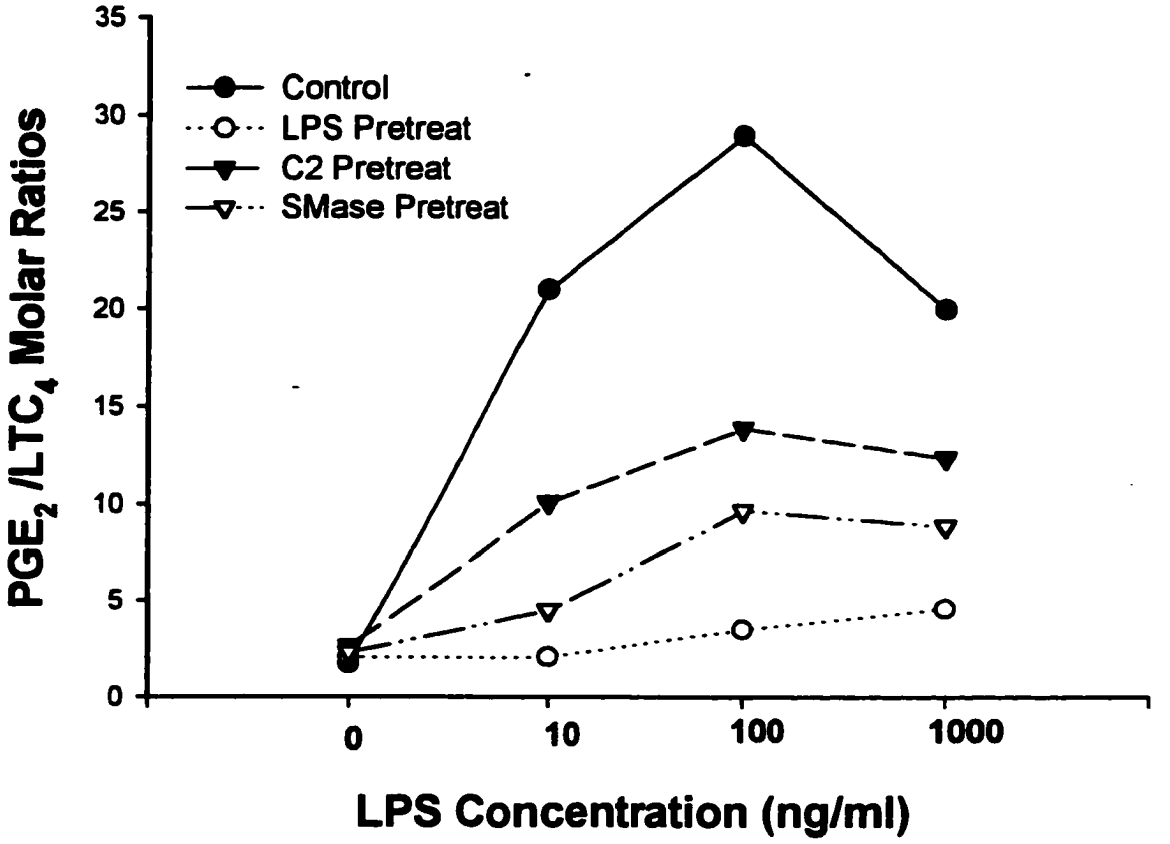


Figure 8. Pre-exposure to LPS, C2 ceramide, or SMase reduced the PGE_2/LTC_4 molar ratios released from THP-1 cells challenged with LPS. THP-1 cells were pretreated as in Fig. 4, and labeled overnight. The cells were then washed and challenged in RPMI with LPS at concentrations shown. Supernatants were assayed for PGE_2 and LTC_4 by EIA, following the procedure provided with the assay kits. Ratios in pg/ml from a standardized curve for averaged duplicate samples were converted to molar ratios.

DISCUSSION

Recent studies have shown that TNFα, IL1β, and LPS share many biological effects and activate common kinases and transcription factors such as CAPK, MAP kinase, and NF-κB (51,104). However, LPS does not activate sphingomyelinase or cause accumulation of ceramide (52). Based on these observations, researchers (52,107) have suggested that LPS may simply mimic ceramide itself. Other observations which appear to be consistent with this hypothesis include the inability of cells from LPS-hyporesponsive C3H-HeJ mice to respond to ceramide (8), the structural similarity between ceramide and the reducing glucosamine of lipid A (52), and the activation of a subset of LPS-inducible genes by the sphingomyelinase pathway (7).

LPS, C2 ceramide, and SMase all activated arachidonic acid metabolism as measured by the release of ^{3}H-AA. At low concentrations of both LPS and ceramide, simultaneous challenge with both agents enhanced the response of THP-1 cells and resulted in an additive to slightly synergistic effect. At higher concentrations of LPS, however, this effect was lost, and the amount of ^{3}H-AA released was reduced below the levels seen with LPS alone (Table 1). If LPS and ceramide were using separate pathways for arachidonic acid metabolism, we expected to see an additive effect at all concentrations since the amounts released through each pathway would be independent of the other. However, loss of this effect at higher concentrations may provide evidence of a shared pathway or signaling component that can be saturated.

These results, simply measuring the release of labeled arachidonic acid metabolites, are consistent with the mimicry model. However, it is important to look at specific

eicosanoids released because studies in our lab have shown that free arachidonic acid accounts for the major portion (approximately 65%) of the label released from treated cells (R. Wang thesis, UM, 1997), so that variations in the relative amounts of the other metabolites would not be apparent. We were able to show that although both LPS and ceramide activated ^{3}H-AA release, they induced very different ratios of PGE_2 to LTC_4. As shown previously (33,76,105), LPS activated the synthesis of prostaglandins, but not leukotrienes, in a concentration-dependent manner. Treatment with SMase also stimulated PG synthesis and high PGE_2/LTC_4 ratios. However, ceramide-treated cells showed slight increases in both PGE_2 and LTC_4, maintaining a stable ratio near constitutive levels. This may simply be due to increased PLA_2 activity and available arachidonic acid, although most of the AA was not metabolized further. These results suggest that for induction of arachidonic acid metabolism, exogenous SMase more closely mimics LPS action than C2 ceramide. The reason for this is unclear. However, exogenous SMase acts at the outer leaflet of the membrane and may release ceramide from a different pool of sphingomyelin so that it has different signaling properties than C2 ceramide or ceramide released by endogenous SMase (109).

For several effects of ceramide (as second messenger for TNFα and IL-1 receptors) and LPS, a common target appears to be ceramide-activated protein kinase (CAPK) which leads to activation of MAP kinases and the translocation of activated NF-κB (52). CAPK may also be involved in the release of eicosanoids. In fact, Schutze (80) has shown that the TNF receptor has two catalytic domains. One domain activates an acid sphingomyelinase and leads to NF-κB activation, and another domain activates a neutral

sphingomyelinase which leads to arachidonic acid release by PLA_2 via CAPK. Jayadev (51) showed that LPS activated CAPK in HL-60 cells at low concentrations and that it was mediated by CD14. Higher concentrations of LPS are able to activate some signaling pathways independent of CD14, which suggests that, like ceramide, there are at least two mechanisms by which LPS activates signal transduction. It seems possible, therefore, that LPS may mimic ceramide inasmuch as it can activate a common target, CAPK, but that some of its activities occur without this shared mediator (Fig. 9).

Additional support for a model in which LPS and ceramide use separate pathways for arachidonic acid metabolism comes from our desensitization studies. Pretreatment of THP-1 cells with LPS desensitizes cells to subsequent challenge with LPS even up to 1000 ng/ml. However, pre-treatment with LPS did not affect the release of label after challenge with C2 ceramide. Pretreatment with C2 ceramide had little effect on LPS challenge, but primed them for release following SMase challenge. Like desensitization, priming of immune cells for subsequent challenges is an important part of the regulation of the immune response (26,74), and results in a much higher release of mediators than that seen without the pretreatment. Because the response of THP-1 cells to LPS differs so dramatically from their response to ceramide treatment, our results suggest that LPS and ceramide do not share a single regulatory pathway. It is possible that LPS may use another membrane target containing a regulatory mechanism that is independent of the CAPK pathway. It is also possible that these results are due instead to an autocrine effect by the high levels of prostaglandins released by LPS-treated cells. PGE_2 has been implicated in the autocrine down-regulation of nitric oxide synthase and tumoricidal

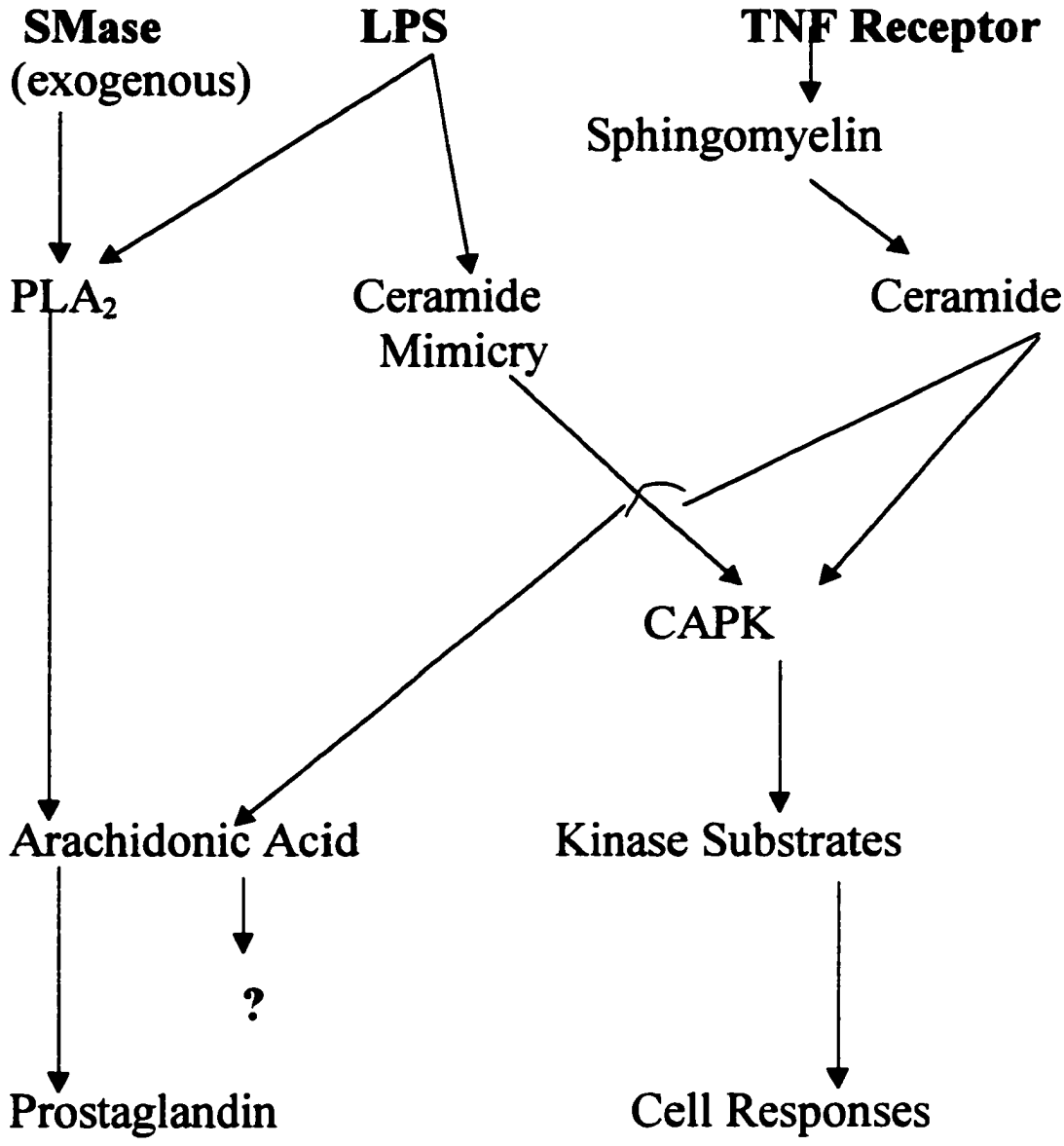


Figure 9. A hypothetical scheme of signaling pathways for arachidonic acid metabolism activated by LPS or ceramide, and the putative shared pathway of ceramide mimicry by LPS. Compiled from various works, as sited in the text.

activity in mouse macrophages (76), and of TNF in a human monocytic cell line (41). If a high PGE_2/LTC_4 ratio is required for desensitization, it may explain why LPS, but not ceramide, is able to desensitize cells to subsequent challenge. It is interesting that SMase, which also caused the release of high PG/LT ratios, was also able to partially desensitize cells to subsequent challenge.

Our data strongly suggest that there are significant differences in the signaling pathways for LPS and ceramide in terms of specific eicosanoids released and in terms of regulation by desensitization. Because of the critical role prostaglandins and leukotrienes play in modulating the immune response, these differences are particularly important. Perhaps even more interesting is our evidence that C2 ceramide and exogenous sphingomyelinase had very different effects. Continuing studies are needed of the specific pools of sphingomyelin from which ceramide is released endogenously by various agents. This will then allow us to explore the possibility of separate pools of arachidonic acid subsequently released as the substrate for cyclo-oxygenase, located in the endoplasmic reticulum, and soluble 5-lipoxygenase. There is evidence that this kind of compartmentalization allows for the production of prostaglandins, but not leukotrienes, by LPS (50).

CHAPTER 5

MONOCLONAL ANTIBODIES TO CD45 MODIFY LPS-INDUCED ARACHIDONIC ACID METABOLISM IN MACROPHAGES

Introduction

Considerable evidence has accumulated that macrophage activation by LPS occurs via a receptor complex consisting of a receptor (CD14) and associated tyrosine kinases of the src family (32,43,60,98). Signaling through CD14 has several similarities to the signaling through the T cell and B cell receptors. These include 1) a requirement for a means to transmit the message into the cell due to the lack of a cytosolic tail on the receptor, 2) activation of protein tyrosine kinases of the src family, and 3) proliferation of multiple signals. The initial dramatic increase in tyrosine phosphorylation is transient (99), which suggests that a regulatory mechanism removes the phosphate groups. It seems likely that this would be a protein tyrosine phosphatase (PTPase) activity.

CD45, the Leukocyte Common Antigen, is a transmembrane protein tyrosine phosphatase (PTPase) that plays a major role in signal transduction in T cells, B cells, natural killer cells, and neutrophils (44,58,61,62,87). It is the most abundant and well characterized of the family of transmembrane protein tyrosine phosphatases in hematopoietic cells (2,58). The molecule (Fig. 1) consists of three domains: a large, extensively-glycosylated extracellular N-terminal domain, a single transmembrane domain, and a cytosolic C-terminal domain that contains two protein tyrosine phosphatase activities (86,89). One of these activities appears to be an autoregulatory protein tyrosine

phosphatase (102). Alternative splicing of three variable exons encoding portions of the extracellular domain leads to expression of several isoforms of CD45. These range in molecular weight from 180 – 240 kD, and their expression is tightly regulated during development and activation of lymphocytes (93).

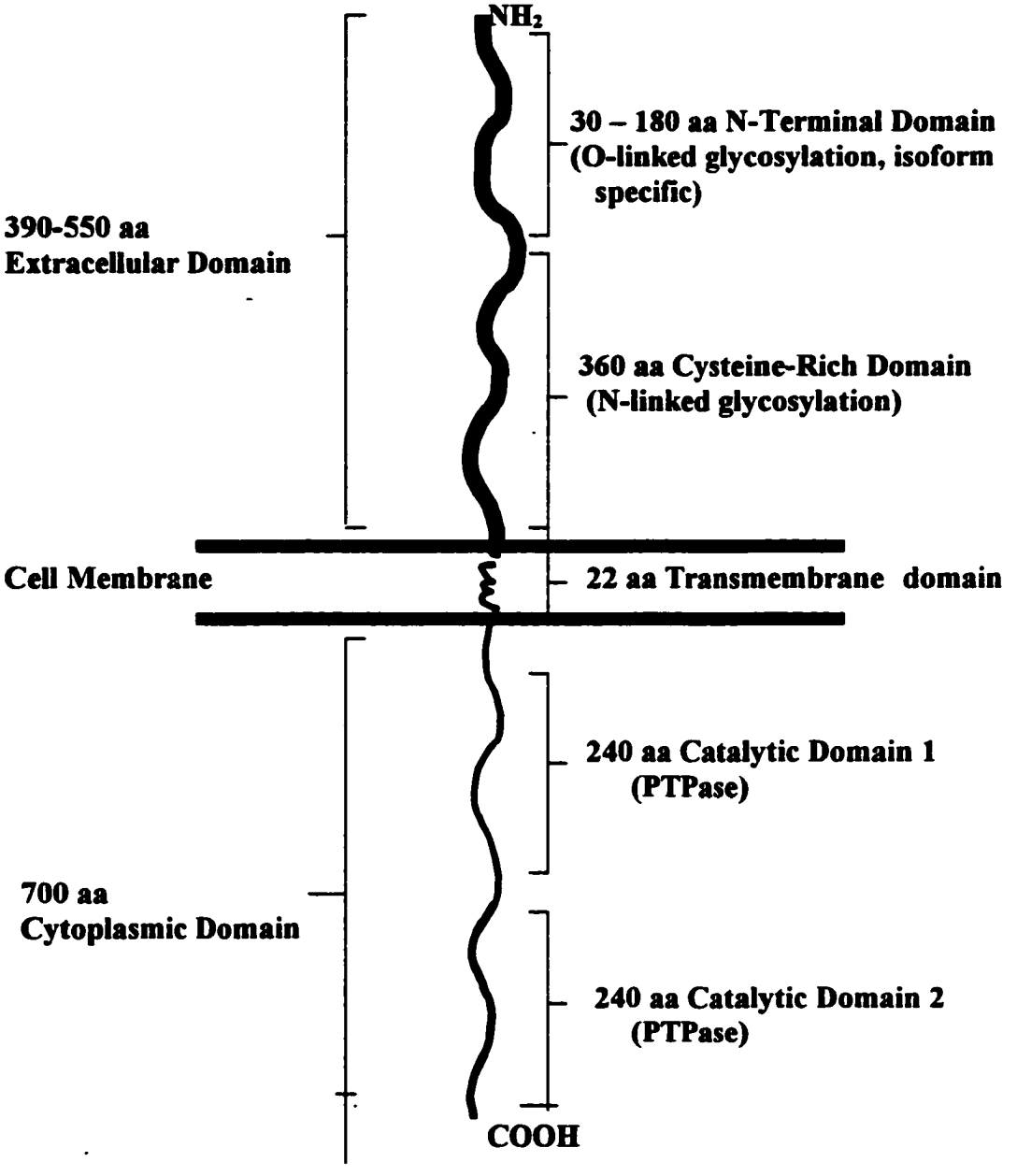


Figure 1. CD45 protein tyrosine phosphatase (Leukocyte Common Antigen)

Although found on monocytes and macrophages (39,61,64,77), CD45 has primarily been studied in lymphocytes. Such studies have demonstrated that CD45 is part of the receptor complexes on T and B cells, in which its major substrates for dephosphorylation appear to be protein tyrosine kinases (PTK) (2,15,87). This suggests that CD45 has a regulatory role in proximal signal transduction by modulating the phosphorylation - dephosphorylation equilibrium, thereby coupling specific surface receptors to downstream tyrosine kinase-dependent signaling. CD45 is involved in both positive and negative regulation depending on whether the particular kinase substrate has phosphorylated tyrosines at inhibitory sites or activation sites (93).

Studies regarding the roles of CD45 in monocytes and macrophages are much more limited. CD45 expression has recently been shown to be up-regulated on activated epithelium-lining macrophages (94), and C5a-activated monocytes (23). There is some evidence for CD45 involvement in monocyte adherence (64), in synergizing with LPS in triggering M-CSF production (39) and respiratory burst in monocytes (61), and in regulation of Fcγ receptor-induced calcium mobilization (53,77). Thus, it is clear that CD45 may be involved in several aspects of macrophage activation. However, very little is known about the role of CD45 in the activation of macrophages by LPS. Since activation by LPS via CD14 results in tyrosine phosphorylation (60), this pathway is most likely regulated by a protein tyrosine phosphatase. Although tyrosine phosphorylation has been associated with eicosanoid biosynthesis in macrophages (33), there have been no studies linking CD45 to the regulation of arachidonic acid (AA) metabolism, another important result of macrophage activation. B cells have been shown to be able to respond

to LPS independent of CD45 (55), but it is possible that this is via a CD14-independent pathway such as polyclonal linkage of surface IgM.

The major objective of this study was to determine whether or not the CD45 protein tyrosine phosphatase plays a role in the activation and desensitization of arachidonic acid metabolism by LPS in THP-1 cells. The hypothesis was based on the role of CD45 in T cells and the apparent similarity between that signaling system and the one used by LPS via CD14, including the evidence of a receptor complex, activation of tyrosine kinases, and the transient nature of the tyrosine phosphorylation induced by ligand binding. Anti-CD45 monoclonal antibodies (mAb) to CD45 were used to explore expression of CD45 on THP-1 cells, to measure their effect on activation and desensitization of AA metabolism by binding or cross-linking surface CD45, and to correlate these effects with signaling through the CD14 pathway.

MATERIALS AND METHODS

Materials: THP-1 cells were purchased from American Type Culture Collection (Rockville MD). Balb/C and Swiss Webster mice were obtained from Jackson Laboratories. Cellgro RPMI 1640 was from Mediatech (Herndon VA), and defined fetal bovine serum (FBS) was purchased from HyClone (Logan UT). Purified LPS R595 from *S. minnesota*, monophosphoryl lipid A (MLA), and diphosphoryl lipid A (DPL) were provided by Ribi Immunochem Research (Hamilton MT). Tritiated arachidonic acid was purchased from American Radiolabeled Chemical Co. (St. Louis MO). Vitamin D3 (1α, 25-dihydroxyvitamin D) was purchased from Calbiochem (LaJolla CA). Anti-CD45, anti-CD45RA, anti-CD45RO, and MØ-P9 anti-CD14/FITC monoclonal antibodies and

isotype-matched negative controls were from Becton-Dickinson Immunocytometry Systems. Anti-mouse anti-CD45 monoclonal antibodies (HI-30) were from Pharmingen (San Diego CA). Enzyme Immunoassay Kits were obtained from Cayman Chemical Co. (Ann Arbor MI). Anti-CD45 (BRA55 biotin-conjugated), anti-CD14 (UCHM-1 biotin conjugated), isotype matched controls, anti-phosphotyrosine (biotin-conjugated), avidin, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis MO).

Cell Culture. Murine peritoneal macrophages were harvested from mice by peritoneal lavage using 10 ml RPMI with 10 μg/ml gentimicin injected into the peritoneal cavity. Cells were plated to 24-well plates and, after a two hour adherence period, non-adherent cells were washed off, and fresh medium was added. THP-1 cells were grown in RPMI supplemented with 10% FBS, 100 U penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine, and maintained between 2 – 6 x 10^5 cells/ml at 37° C with 5% CO_2. Viability remained above 97% (by trypan blue exclusion) throughout the experiments except where noted. THP-1 cells were pretreated for 24 hours with 0.1 μM vitamin D3 to induce expression of CD14, the putative LPS receptor and marker for a more mature (macrophage) phenotype. They were then labeled with ^{3}H-arachidonic acid at 0.25 μCi/ml overnight.

Cell Activation. Prior to challenge the cells were washed with warm RPMI, resuspended in 25 ml of RPMI, and transferred to 24 well culture plates (1 ml/well). The final heat-inactivated serum concentration was 2.5 – 5%, and final cell densities were between 5 – 8 x 10^5 cells/ml. LPS was solubilized in 0.1% triethylamine in a 100 mg/ml stock solution. Monoclonal antibodies were titered for use at approximately 2 μg/ml for

10^6 cells, and diluted in RPMI. Controls were included to rule out effects of the carriers alone. Isotype-matched anti-myeloma protein mAb were used to rule out effects of binding by FcR on the macrophages. Endotoxin levels of vitamin D3 and media solutions were shown to be below detectable levels by Limulus assay.

At the end of challenge time periods, samples were removed from the wells, cells were pelleted by centrifugation, and 200 μl of the supernatant were counted in a Beckman scintillation counter to determine the amount of radiolabeled arachidonic acid metabolites released. Supernatants were also assayed for specific eicosanoids (prostaglandin E_2, leukotriene B_4, and leukotriene C_4) using EIA kits, following manufacturer's instructions (Cayman).

Desensitization. Cells were treated with 100 ng/ml LPS (R595), with or without anti-CD45, for 3 hours and then washed and resuspended in fresh RPMI with 10% heat-inactivated FBS prior to the overnight labeling with ^{3}H-arachidonic acid (^{3}H-AA).

Cross-linking of surface receptors. Cross-linking of CD45 and CD14 was accomplished in two ways. First, cells were treated with Anti-CD45 2D1 for 10 min at 1 μg/ml, then washed and plated to wells in 24-well plates at $5x10^5$ cell/ml. Anti-mouse IgG was added at 1 μg/ml to cross link the bound mAb prior to challenge. Second, cells were treated as above with biotinylated anti-CD45. Cells were then washed to remove free mAb. Avidin was then added at 30 μl/ml prior to challenge.

Flow cytometry. After overnight treatment with LPS R595, MPL, or C2 ceramide, D3 pretreated THP-1 cells were stained with FITC or phycoerythrin (PE) conjugated monoclonal antibodies to CD45, CD45RA, and CD45RO from Becton-

Dickinson. The staining period was 30 min. at 4° C. Cells were washed twice with staining buffer (PBS, 0.05% sodium azide, 2% FBS) and fixed with 1% buffered paraformaldehyde. PE and FITC conjugated isotype-matched negative control myeloma proteins were used throughout to quantitate nonspecific background binding to target cells. Forward angle (FALS) and 90° (orthogonal) light scatter gating eliminated debris and dead cells from the analysis on a Coulter Elite flow cytometer. Data represent the percentage of positively stained cells for a given antibody, projected beyond the cursors set on negative control histograms to separate positively and negatively stained cells. Cursors were set so that 1-2% of all cells stained with the negative control, isotype-matched reagents were displayed as being in the positive region of the histogram.

SDS-PAGE and Western Blotting. D3-pretreated THP-1 cells (1×10^7) were lysed in 1 ml lysis buffer for 1 hr at 4° C. (Lysis buffer = 1% Triton X100, 20mM Tris HCl (pH 7.4), 137 mM NaCl, 2mM EDTA, 1mM phenylmethylsulfonylfluoride (PMSF), 1 μg/ml each of pepstatin, aprotinin and leupeptin, 1 mM sodium orthovanadate ($NaVO_4$), and 100 μM phenylarsine oxide (PhAsO).) The lysate was pelleted at 10,000 g for 30 min, and supernatants were transferred to fresh tubes. For immunoprecipitation, 20 μl of anti-CD45 was added and incubated for 2 hours at 4° C. 50 μl of protein-G sepharose was then added and incubated at 4° overnight on an inverter shaker. Precipitate was washed twice in lysis buffer, and once in wash buffer (50 mM Tris HCl 7.4, 200 μM $NaVO_4$, 100 μM PhAsO). Samples were boiled for 5 min prior to loading onto 7.5% SDS-PAGE gels. Gels were run in a BioRad Minigel apparatus for 40 – 60 min, then blotted onto PDVF membranes. Blots were stained with primary antibodies or with

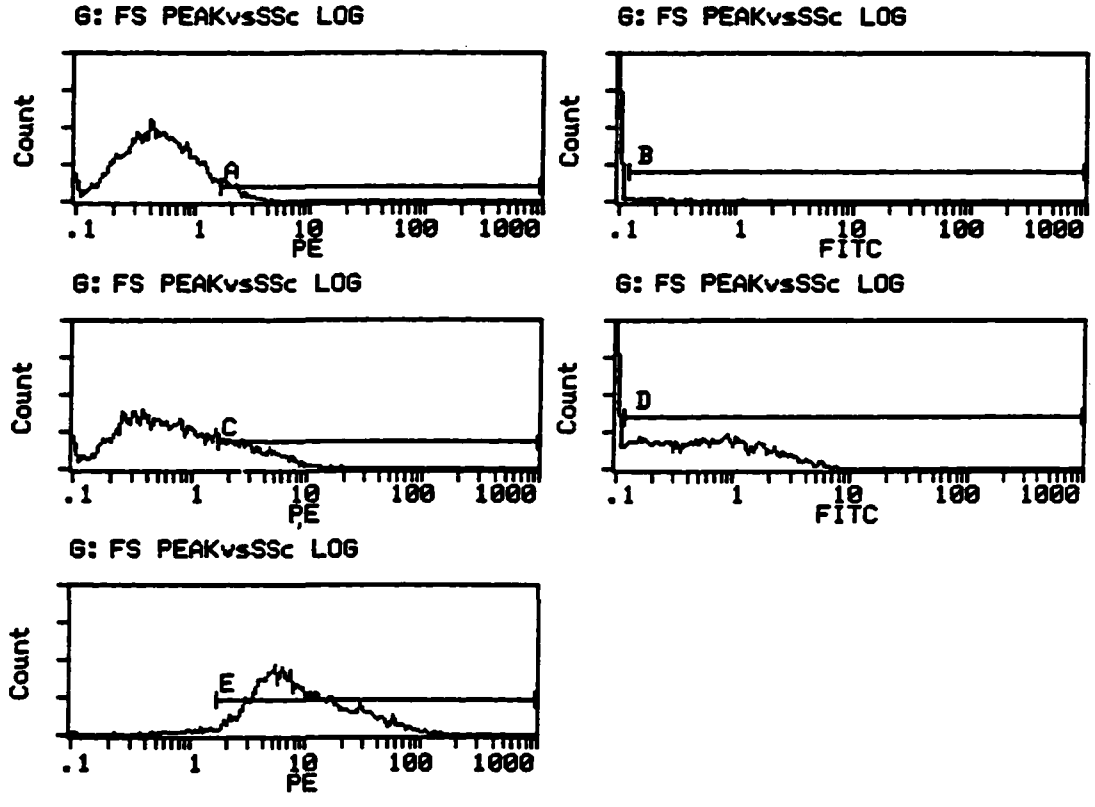
biotinylated Abs for 2 hours at room temperature in blocking buffer, then washed for 30 min. If needed, biotinylated 2° antibody was used to stain the primary Ab, and the membrane was washed again. Blots were stained for 1 hour at room temperature in blocking buffer with streptavidin:horseradish peroxidase, then washed 15 min with 60° wash buffer, and subsequent washes at 37° for 15 min and 5 min. Blots were developed and films exposed using enhanced chemiluminescence (ECL) kits from Amersham.

RESULTS

THP-1 cells express CD45 constitutively. Figure 2 shows flow cytometry histograms from cells stained with three different monoclonal antibodies (mAb) specific for three of the CD45 isoforms commonly expressed on leukocytes. Each histogram has a peak representing the percent of cells in the gated population that stained positively with the indicated antibody. The position of the peak on the X axis shows mean channel fluorescence, a measure of how brightly the cells are stained. The first two histograms show staining with negative control, (IgG_1/IgG_{2a}) conjugates, phycoerythrin (A) and FITC (B). The cursors are set on these controls so that only a small percentage (0.1 – 1.6%) of the cells staining with the negative controls are counted as positive. Histogram C shows that 24.5% of the gated cells stained with anti-CD45RO/PE with a mean fluorescence of 3.52. Generally, CD45RO is the isoform expressed on activated T cells, and CD45RA is more often expressed on quiescent cells. Histogram D shows 63.1% of the cells stained positive with anti-CD45RA/FITC with a mean fluorescence of 0.628. This means that although more cells expressed CD45RA, there were significantly fewer molecules

expressed/cell than CD45RO. Histogram E shows 95.7% of the cells stained positive for anti-CD45, a mAb that recognizes all known isoforms of CD45, at a mean fluorescence of 9.52.

This pattern did not change significantly following treatment with LPS, MPL, or ceramide (Table 1), suggesting that activation of macrophages by these agents does not alter expression of CD45 isoforms. There was a 50% reduction in % positive cells stained for isoforms RO and RA after treatment with C2 ceramide. Little is known about the regulation of CD45 isoforms on macrophages, and I did not explore this using THP-1 cells since it would have required comparing expression on these cells with several other cell systems. The alteration in expression following C2 treatment was intriguing, however, and would make another interesting study.



STATISTICS

SINGLE PARAMETER STATISTICS

ID	Pcnt	Area	Peak Position	Peak Height	X Channel Mean	X Channel SD	X Channel FullCV	X Channel HalfCV	X Channel Min	X Channel Max
A	7.2	358	1.7	12	2.42	1.14	47.2	1.43	1.6	1024
B	1.9	93	0.14	3	0.311	0.283	91.2	0.668	0.12	1024
C	24.5	1225	1.7	19	3.52	2.37	67.3	0.930	1.6	1024
D	63.1	3154	0.81	18	0.628	0.699	111.3	0.494	0.12	1024
E	95.7	4783	5.4	30	9.52	9.18	96.4	12.3	1.6	1024

A Low Channel = 1.6, High Channel = 1024 C
B Low Channel = 0.12, High Channel = 1024 B
C Low Channel = 1.6, High Channel = 1024 C
D Low Channel = 0.12, High Channel = 1024 B
E Low Channel = 1.6, High Channel = 1024 C

Figure 2. CD45 expression on THP-1 cells. D3-pretreated THP-1 cells were stained for flow cytometry as described in Materials and Methods. A)IgG_1/IgG_{2a} isotype matched control conjugated with phycoerythrin; B) IgG_1/IgG_{2a} isotype control conjugated with FITC; C) Anti-CD45RO/PE; D) anti-CD45RA/FITC; E) anti-CD45/PE.

TABLE 1

Surface expression of CD45 isoforms on vitamin D3-pretreated THP-1 cells following treatment with medium , LPS, C2 ceramide, or MPL, as measured by flow cytometry.[a]

TREATMENT:	Control	LPS	MPL	Ceramide
αCD45RA	63.1[b]	61.4	53.9	35.7
	0.699[c]	0.6	0.557	0.428
αCD45RO	24.5	27.4	25.2	12.5
	3.52	3.43	3.64	3.3
αCD45 (*pan*)	95.7	97.1	97.8	97.7
	9.52	14.2	11.2	11.3

a) THP-1 cells were treated for 18 hr with medium only (control), 100 ng/ml LPS R595, 1000 ng/ml MPL, or 40 μM C2 ceramide. Cells were then stained and analyzed by flow cytometry as described in Materials and Methods.

b) Percent of gated (90° vs FALS) cells staining positive
c) Mean channel fluorescence – 4 decade logarithmic range

When whole cell lysates were separated by PAGE and Western blotted, anti-CD45 clone BRA55 (Sigma) stained a set of three bands at 240, 200, and 180 kD (Fig. 3a). This suggests that the full range of isoforms sizes are expressed on THP-1 cells, all of which contain the epitope bound by BRA55. Anti-CD45 2D1 (Becton-Dickinson) stained a single band at 220 kD (Fig. 3b).

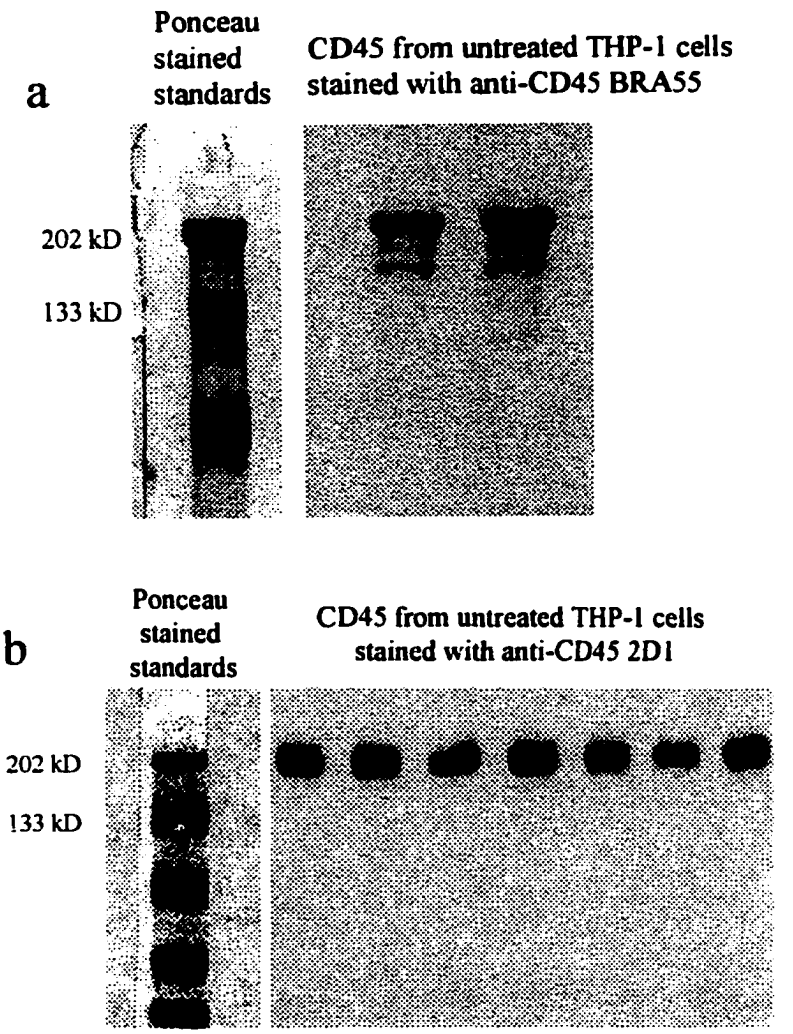


Figure 3. Western blot of CD45 on THP-1 cells. Whole cell lysates of D3-pretreated THP-1 cells in sample buffer were loaded onto 7.5% SDS-PAGE gels and run in a min-gel apparatus for 40 min. Proteins were then blotted onto PDVF membranes. Blots were stained with either biotinylated anti-CD45 BRA55 (a), or anti-CD45 2D1 followed by 2° biotinylated anti-mouse anti-IgG (b), and then streptavidin:HP. Blots were developed and films exposed using ECL.

Anti-CD45 mAb increased the release of labeled arachidonic acid metabolites and TNF from LPS-challenged cells. Addition of anti-CD45 2D1 just prior to challenge with LPS increased the release of Aa slightly (Fig. 4a), but when added 18 hr prior to challenge there was a 2-fold increase over the amount released from cells

challenged only with LPS (Fig. 4b).

Similar results were seen when mouse peritoneal cells (PECs) were treated with anti-mouse αCD45 18 hours prior to challenge (Fig. 5). Like the increase seen with THP-1 cells, αCD45 resulted in at least a doubling of the amount of ^{3}H-AA released.

Macrophages express Fc receptors (FcR) for binding particles opsonized with antibodies. Binding of FcR can lead to activation of several pathways (21,77). To determine whether FcR activation was a factor in the increased arachidonic acid release, THP-1 cells were treated with isotype matched control antibodies for 2 hours. Figure 6a shows that although LPS and anti-CD14 UCHM-1 activated AA release, anti-CD45 2D1 had only a slight effect by itself. IgG_1 (isotype control for 2D1) and IgG_{2a} (isotype control for UCHM-1) had no effect. Fig. 6b shows that the isotype matched negative control IgG_1 did not have any effect on LPS challenge.

Anti-CD45 2D1 affected a signaling pathway activated by LPS other than arachidonic acid metabolism, that leading to TNFα production. It had no effect alone on TNF production, but synergized with LPS to release 33-42% more TNF from cells treated with 1-10 ng/ml of LPS, respectively (Fig. 7).

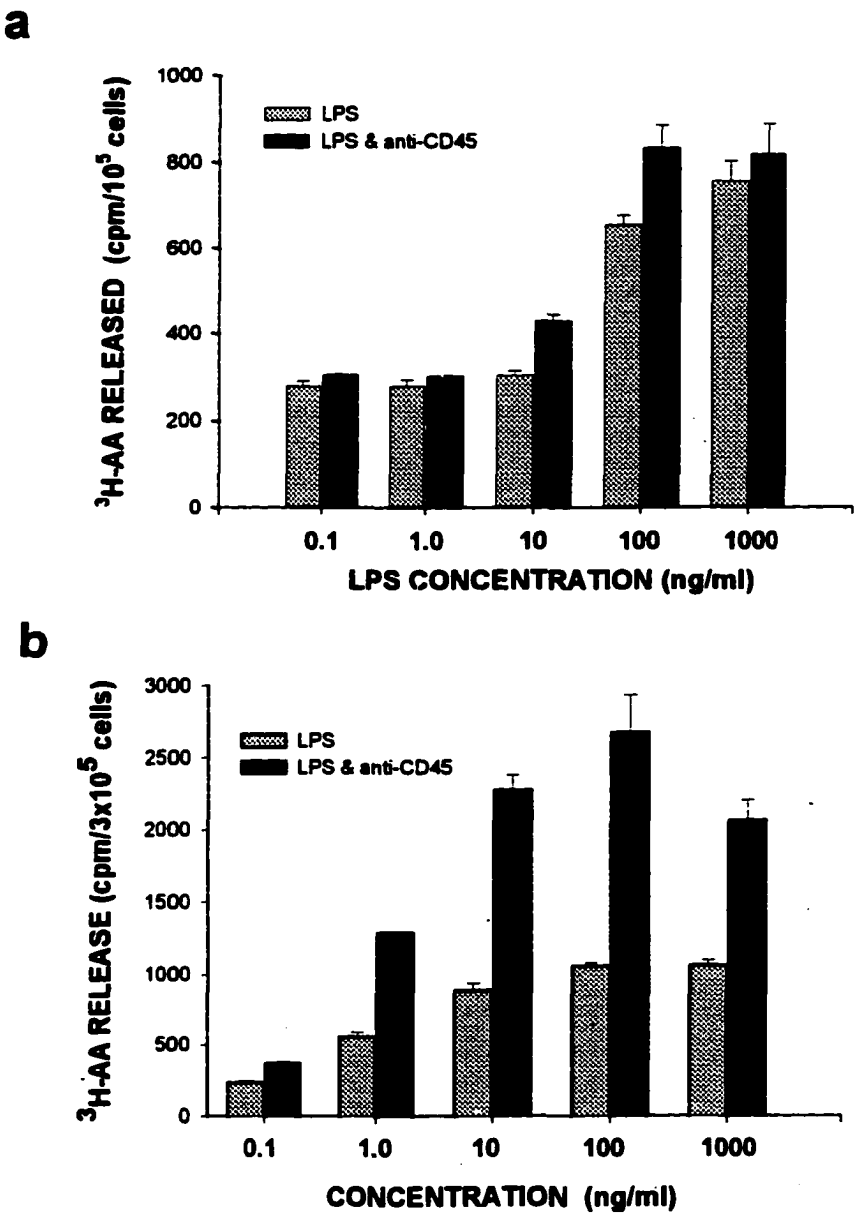


Figure 4. Anti-CD45 mAb increased ^{3}H-AA release from LPS-challenged THP-1 cells. D3-pretreated THP-1 cells were labeled overnight with 0.5 μC/ml ^{3}H-AA, then washed and resuspended in RPMI for plating in 24-well plates at 1.5x10^6 cells/well. αCD45 2D1 was added either immediately prior to challenge (a), or 18 hours prior to challenge (b). The cells were challenged with LPS R595 at concentrations shown for 3 hr. Supernatants were centrifuged to remove cells, and 200 μl of supernatants were counted for cpm released. Values represent cpm released from 3x10^5 cells above control values for cells challenged with medium alone, and are averages of duplicates ± range.

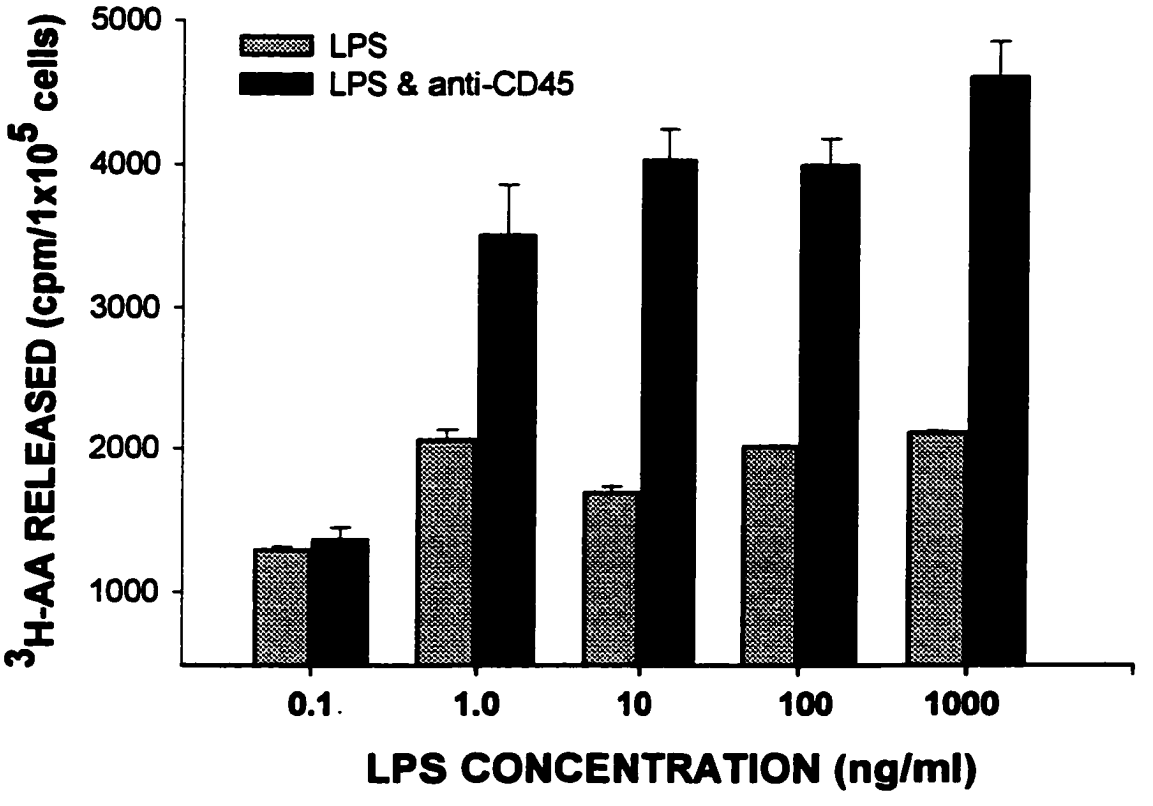


Figure 5. Anti-CD45 increased ^{3}H-AA release from LPS-challenged mouse peritoneal exudate cells (PECs). PECs were harvested from 6-week old Balb/C mice and plated in 24-well plates. After a 2 hr adherence period, non-adherent cells were removed by washing, and adherent cells were labeled overnight as before, one plate containing anti-mouse αCD45, 1 μg/ml) and one plate with no mAb. Wells were washed and cells were challenged with LPS R595 for 2 hr. Values represent cpm released above control values for unchallenged cells, and are averages of duplicates ± range.

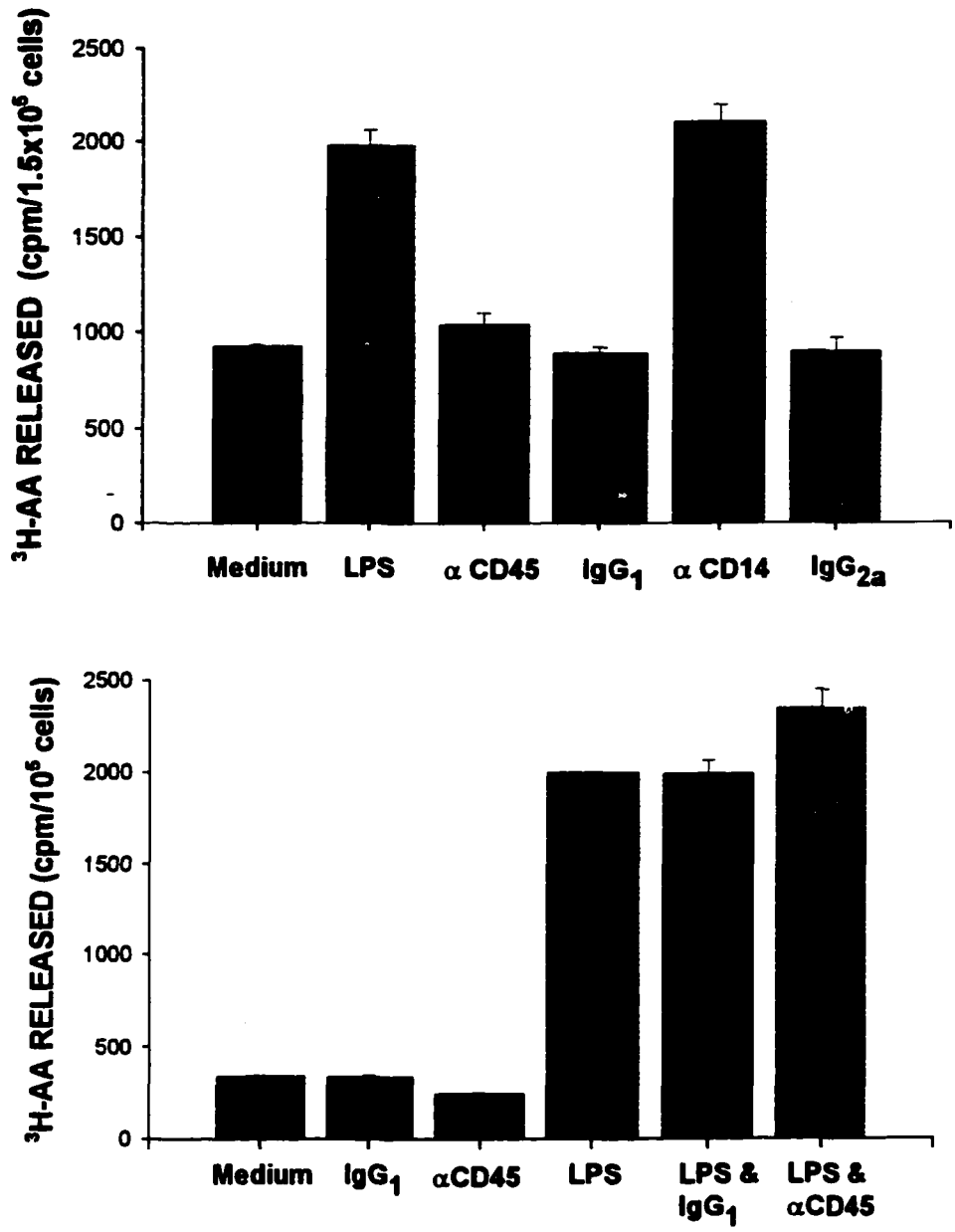


Figure 6. Isotype-matched control antibodies had no effect on AA release. D3-pretreated THP-1 cells were labeled overnight as before, then washed and plated at $5x10^5$ cells/well. LPS (1 μg/ml) or 1 μg/ml of the various antibodies were added to wells. The mAbs used were αCD45 2D1 (Becton-Dickinson, IgG_1), IgG_1 isotype control (Becton-Dickinson), αCD14 UCHM-1 (Sigma, IgG_{2a}), and IgG_{2a} isotype control (Sigma). Cells were challenged for 2 hr, then 200 μl of supernatants were counted for cpm released. Values are averages of duplicates ± range.

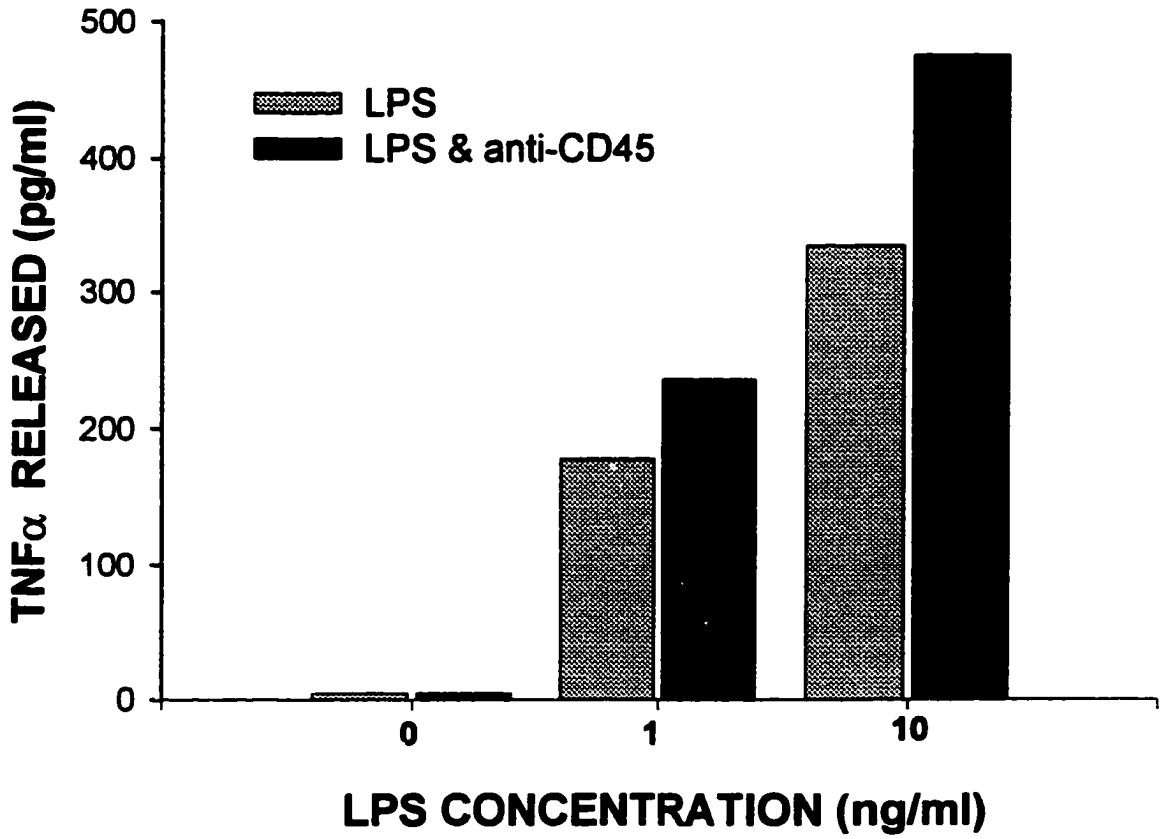


Figure 7. Anti-CD45 increased release of TNFα from THP-1 cells challenged with LPS. Vitamin D3-pretreated THP-1 cells were challenged for 20 hr with LPS R595 at concentrations shown, with or without 1 μg/ml αCD45 2D1. Supernatants were assayed for TNFα released by ELISA according to manufacturer's instructions. TNFα in pg/ml was calculated from a standard curve.

Cross-linking of CD45 inhibited LPS-induced activation of AA release.

CD45 was cross-linked on THP-1 cells using two methods. Anti-CD45 was allowed to bind to cells for 10 minutes prior to the addition of either avidin (to cross-link biotinylated anti-CD45), or anti-mouse IgG (to cross-link the Fc domains of the bound anti-CD45). Figure 8 shows that both methods reduced AA release significantly from that released from LPS-challenged cells. IgG_1 isotype control and the anti-mouse IgG mAb had no effect.

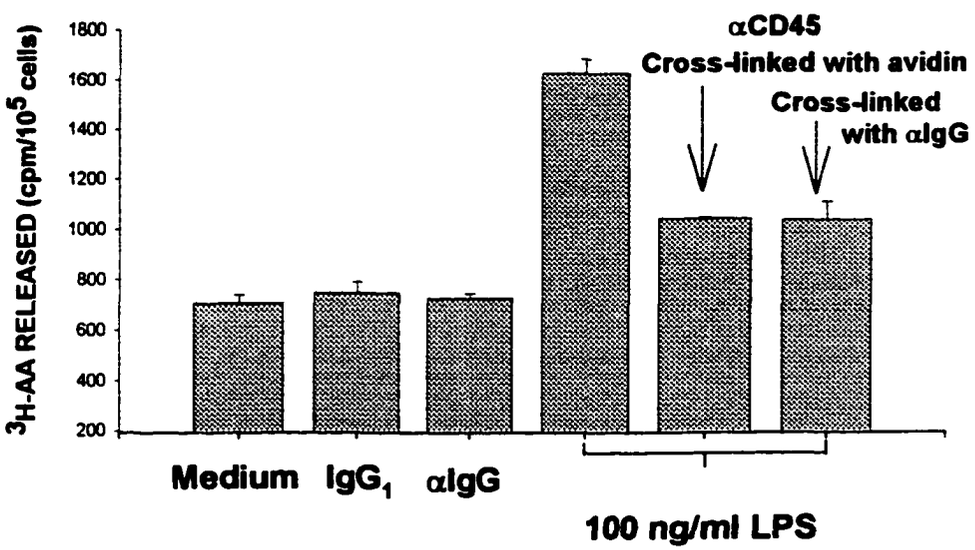


Figure 8. Cross-linking CD45. D3-pretreated THP-1 cells were labeled overnight with ^{3}H-AA , then divided into flasks containing either medium alone of 1 μg/ml of anti-CD45 BRA55. After 10 min to allow binding, cells were washed and resuspended in fresh medium, and plated at $5x10^5$ cells/well. Either anti-mouse αIgG (1 μg/ml) or 20 μl avidin were added to cross-link via Fc domains or biotinylated αCD45, respectively. Cells were then challenged with LPS R595 for 2 hr. 200 μl of cell-free supernatants were counted for cpm released. Values represent relative response, calculated by dividing cpm released from challenged cells by the cpm released from unchallenged cells, and are averages of duplicates.

Desensitization and anti-CD45. As shown in previous chapters, LPS pretreatment dramatically reduces eicosanoid release following subsequent challenge. Figure 9 shows that anti-CD45 added during the desensitization period (2-3 hours) did not affect the ability of LPS to desensitize the cells. However, addition of anti-CD45 during that period without LPS resulted in increased release after subsequent challenge. Therefore, treatment of these cells with anti-CD45 18-20 hours prior to challenge increased release of AA even if the unbound antibodies are washed away after 2 hours, although to a lesser extent than if the mAb were left in overnight.

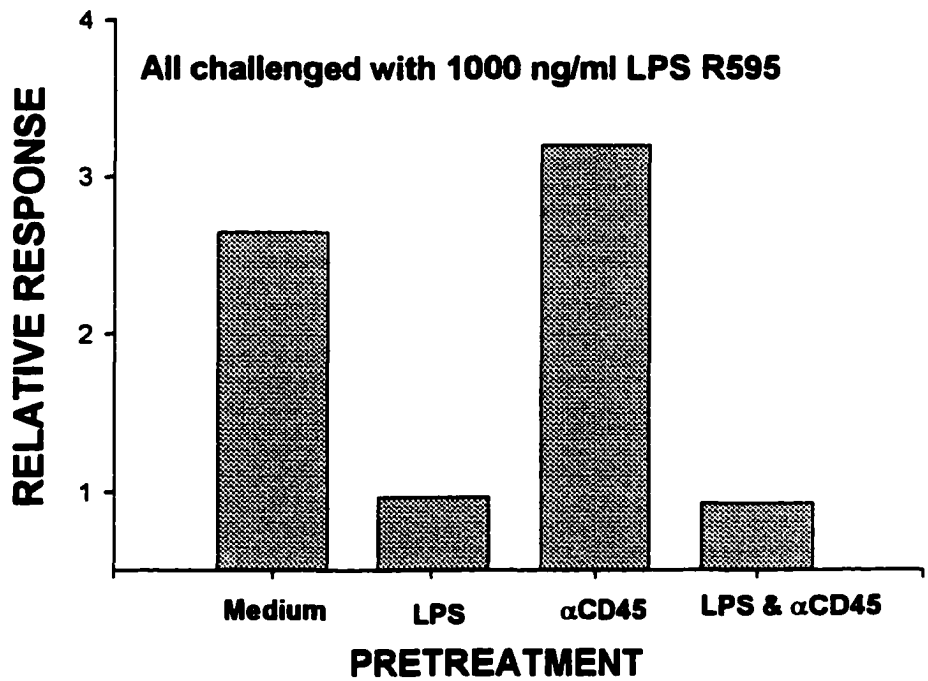


Figure 9. Pretreatment with αCD45 had no effect on desensitization by LPS. D3-pretreated THP-1 cells were treated with medium alone, 100 ng/ml LPS R595, 1 μg/ml αCD45 2D1, or LPS and αCD45 together for 3 hr. The cells were then washed and labeled overnight as before. They were washed and plated at $1x10^6$/well, and challenged with LPS at 1 μg/ml for 2 hr. 200 μl of cell-free supernatants were counted for cpm released above controls, and values are averages of duplicates.

Production of prostaglandin E_2. One of the important eicosanoids released from LPS-activated macrophages is PGE_2 (see Chapter 4). Figure 10 shows that anti-CD45 increased production of PGE_2 from LPS-challenged cells. There was no production of LTC_4 or LTB_4 with either LPS, LPS and anti-CD45, or anti-CD45 alone (data not shown). This suggests that, in terms of prostaglandin and leukotriene production, anti-CD45 simply increased the signal generated by LPS, but did not change which signaling pathways were activated. This is further evidence that the effect seen with anti-CD45 is not due to FcR binding, since activation of signaling by FcRγ has been shown to cause the production of LTB_4 from both monocytes and PMNs (49,83).

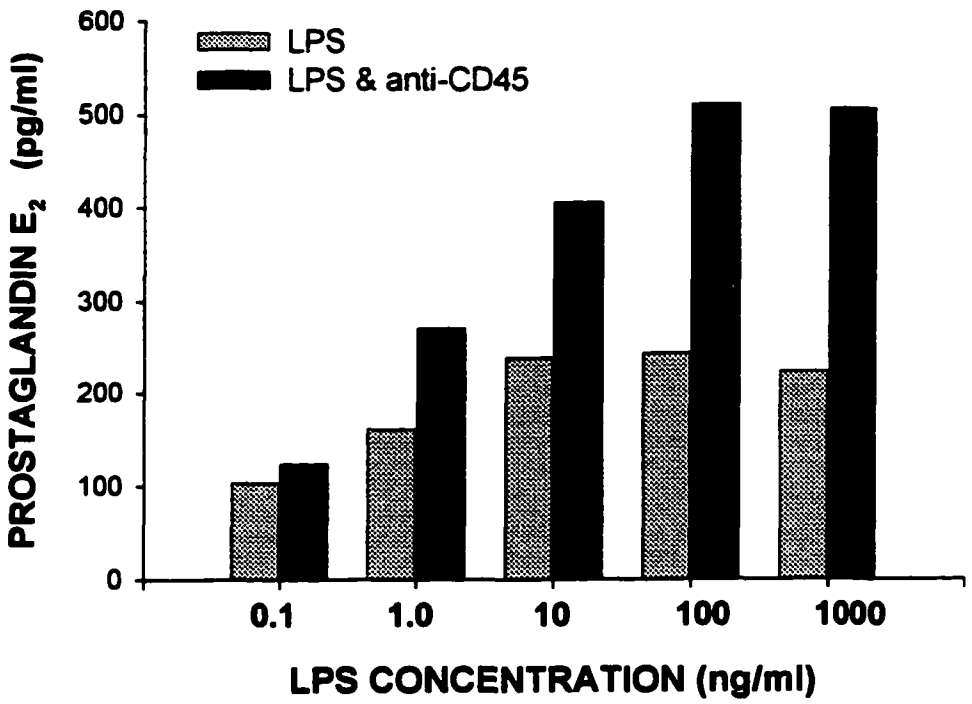


Figure 10. αCD45 increased PGE_2 released from LPS-challenged cells. Samples from Fig. 3 were assayed for PGE_2 using EIA, following manufacturer's instructions. PGE_2 in pg/ml was calculated from a standard curve. Values are pg/ml released above control, and are averages of duplicates.

Correlation of the effects of anti-CD45 with signaling through the CD14 signaling pathway. Because LPS activates tyrosine phosphorylation via CD14 (33), this pathway is likely to have an associated protein tyrosine phosphatase. This series of experiments was done under conditions that emphasized signaling through CD14. CD14 specifically binds complexes of LPS and its serum binding protein, LBP. Therefore, THP-1 cells were challenged in medium without serum to determine the effect of anti-CD45 in the absence of LBP. Rather than the dramatic increase seen in medium containing 5% serum (Fig. 4), a slight reduction in release was seen with anti-CD45 in the absence of serum (Fig. 11).

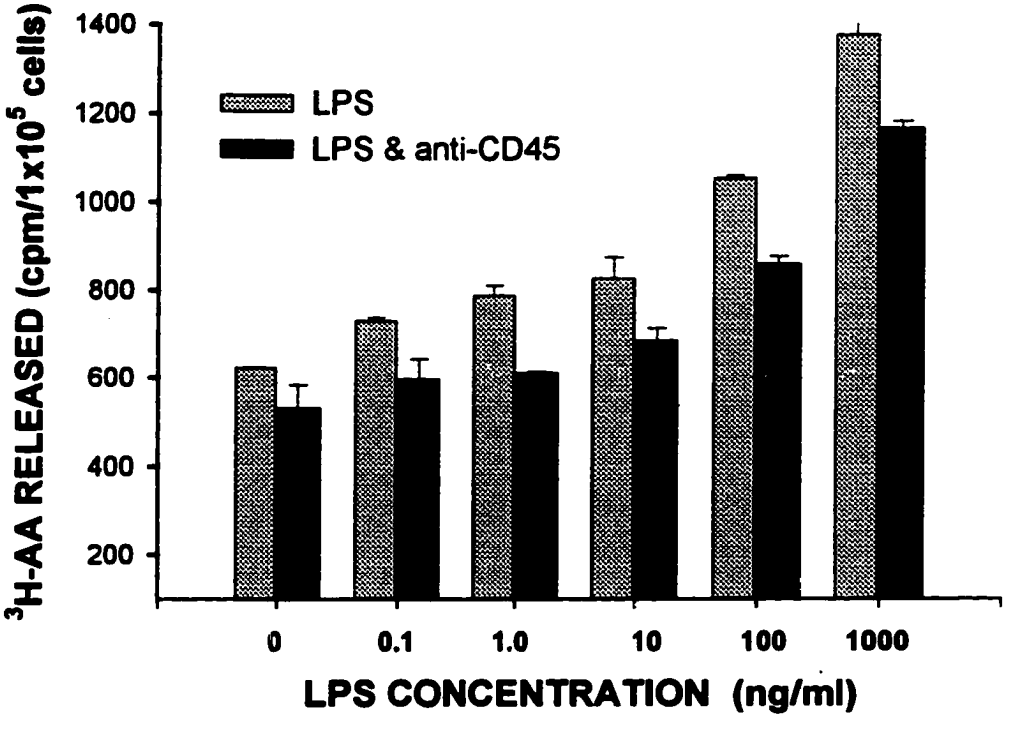


Figure 11. Lack of serum in the challenge prevented the increase in AA release by αCD45. D3-pretreated THP-1 cells were labeled and challenged as in Fig. 3 except that no serum was added to challenge medium. Values represent cpm released in 200 μl of cell-free supernatants, and are averages of duplicates ± range.

Pretreatment of THP-1 cells with vitamin D3 increases CD14 expression 50-80% (Chapter 2, Table 1). Therefore, effects of αCD45 were compared on cells treated with D3 to cells that were not pretreated with D3. Without D3 pretreatment, αCD45 had very little effect except at the highest LPS concentration (100 ng/ml) (Fig. 12). The effect was seen with cells treated with D3 even at the lowest concentration of LPS tested (0.1 ng/ml).

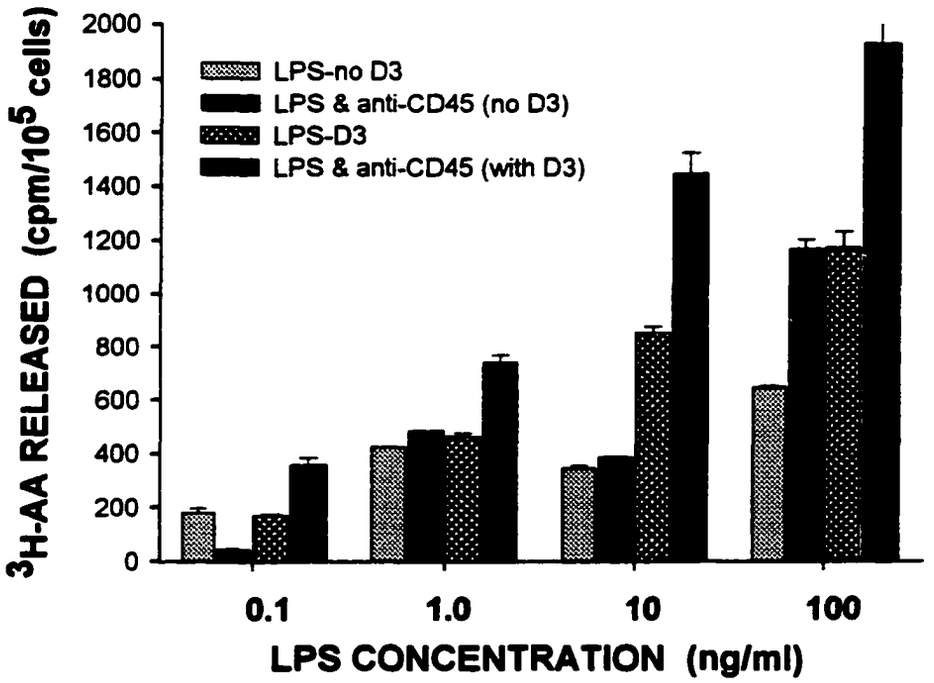


Figure 12. Pretreatment with vitamin D3 increased the effect of αCD45 on AA release. Equal volumes of THP-1 cells at $5x10^5$ cells/ml, either treated or not treated with vitamin D3 (0.1 μM), were labeled and challenged as in Fig. 3. Values represent cpm released in 200 μl of cell-free supernatants above control, and are averages of duplicates ± range.

Several other bacterial amphiphiles have been shown to bind CD14, including MPL, DPL, and LTA (See Chapter 3). Other agents, including C2 ceramide, sphingomyelinase (SMase), and phorbol myristate acetate (PMA), activate AA metabolism independent of CD14 binding. Anti-CD45 increased release from cells treated with all of the CD14 ligands (Figure 13), but none of the CD14-independent agents (Figure 14).

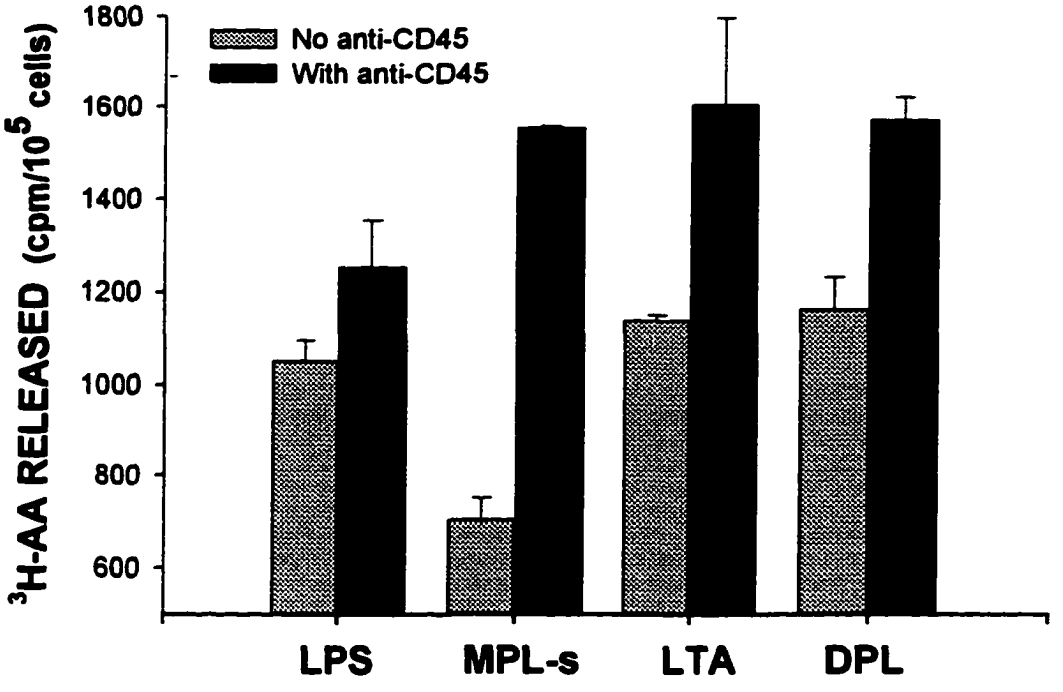


Figure 13. αCD45 affects AA release activated by other CD14 ligands: MPL-s, DPL, and LTA. D3-pretreated THP-1 cells were labeled as in Fig. 3, and challenged with medium alone, or 1 μg/ml LPS R595, MPL-s, DPL, or LTA (*E. faecalis*) for 2 hr. Values represent cpm released in 200 μl of cell-free supernatants above control, and are averages of duplicates ± range. The experiment was repeated 3 times.

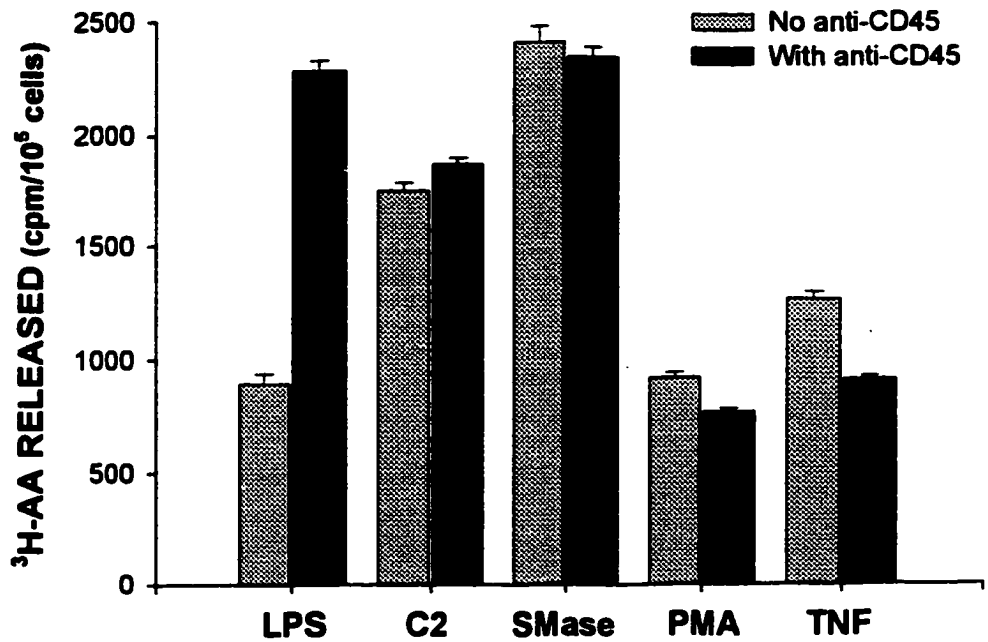


Figure 14. αCD45 did not affect ^{3}H-AA release activated by agents using CD14-independent signaling. D3 pretreated THP-1 cells were labeled as in Fig. 3 and challenged with 50 μM C2 ceramide, 0.5 U/ml SMase, 10 ng/ml LPS for 2 hr, or 20 ng/ml TNF, 30 ng/ml PMA for 5 hr. Values represent cpm released in 200 μl cell-free supernatants above control, and are averages of duplicates ± range. The experiments were repeated 2-3 times.

Figure 15 shows that anti-CD45 also increased production of TNFα from cells treated with MPL-s, one of the CD14 ligands.

Finally, anti-CD14 UCHM-1 activates AA release from THP-1 cells, and may serve as an LPS agonist, directly activating signaling by binding to the LPS-binding site on CD14. Isotype-matched control IgG_{2a} was shown to have no effect on AA release (Fig. 6). To further confirm its role as an LPS agonist, I was able to show that, like LPS, anti-

CD14 UCHM-1 induced only PGE_2 and not LTC_4 production, giving high PGE_2/LTC_4 ratios (Fig. 16), was able to desensitize cells to LPS challenge (Fig. 17), and had very little effect on cells without D3 pretreatment (Fig. 18). Figure 18 also shows that anti-CD45 significantly increased AA release from cells treated with anti-CD14.

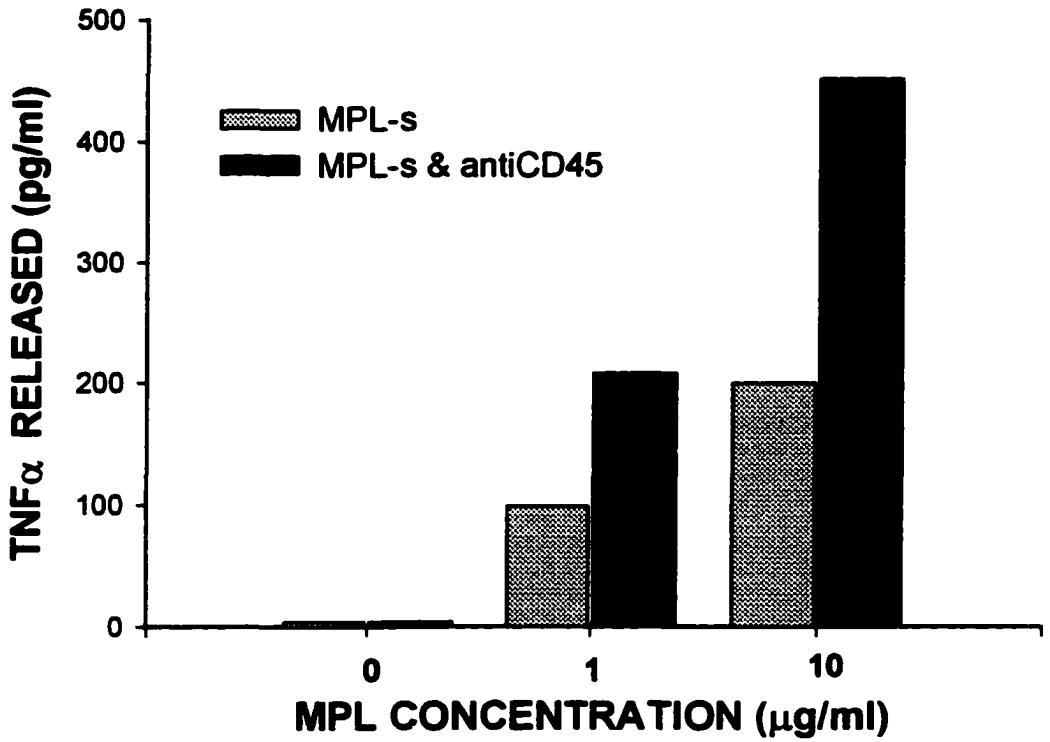


Figure 15. αCD45 increased release of TNFα from THP-1 cells challenged with MPL-s. D3-pretreated THP-1 cells were challenged for 20 hr with MPL-s at concentrations shown, with or without αCD45 2D1. Supernatants were assayed for TNFα by ELISA according to Quantikine instructions. TNFα in pg/ml was calculated from a standard curve.

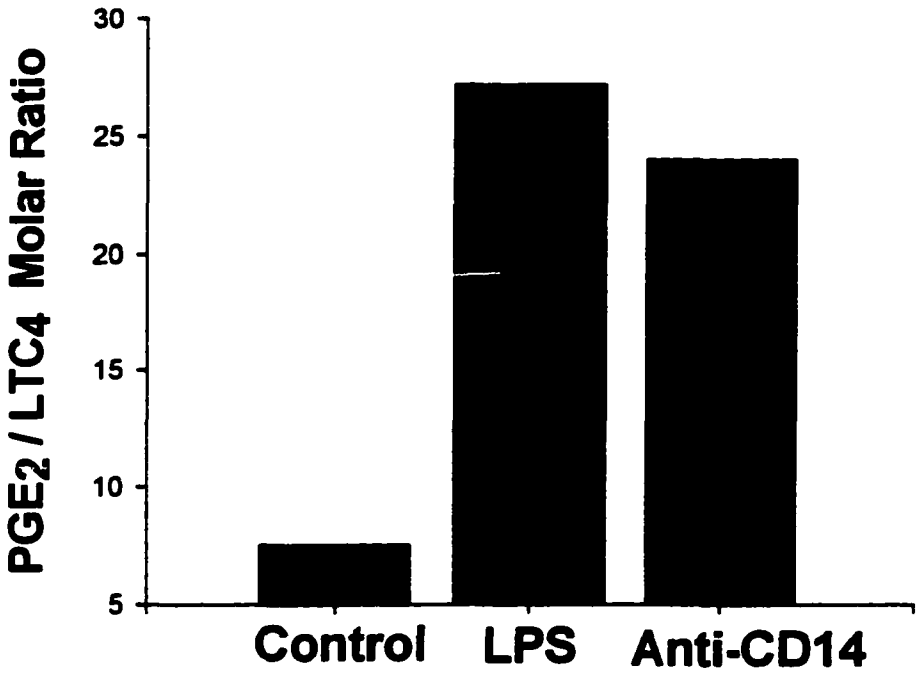


Figure 16. αCD14 UCHM-1 induced PGE_2 production. Supernatants from D3-pretreated THP-1 cells that had been challenged with αCD14 UCHM-1 were assayed for PGE_2 and LTC_4 using EIA, according to manufacturer's instructions. PGE_2 & LTC_4 in pg/ml were calculated from standard curves. Values are molar ratios of PGE_2 to LTC_4, and are averages of duplicates ± range.

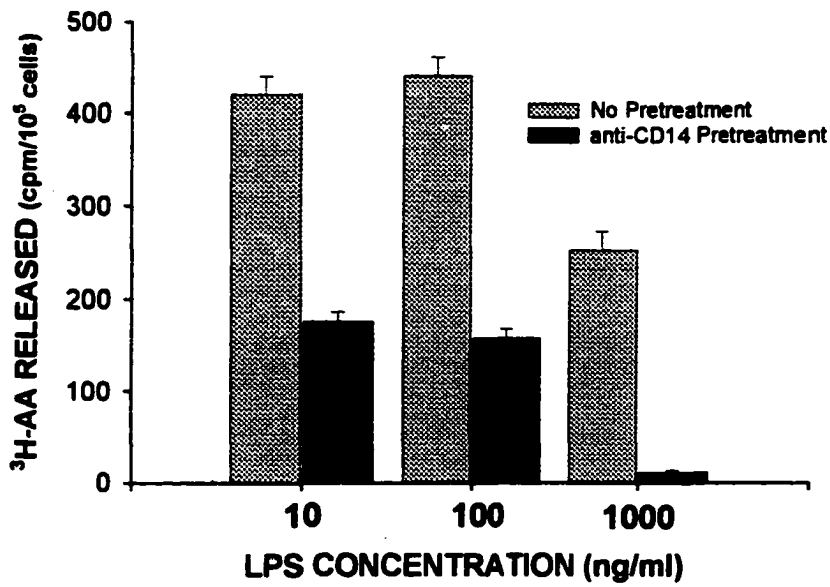


Figure 17. Desensitization of THP-1 cells with αCD14. D3-pretreated THP-1 cells were treated with medium alone or 1 μg/ml αCD14 UCHM-1 for 2 hrs. Cells were then washed and labeled overnight with ^{3}H-AA. They were washed again and challenged in 24 well plates ($5x10^5$ cells/ml) with LPS R595 for 2 hr. 200 μl of cell-free supernatants were counted. Values represent cpm released above control, and are averages of duplicates.

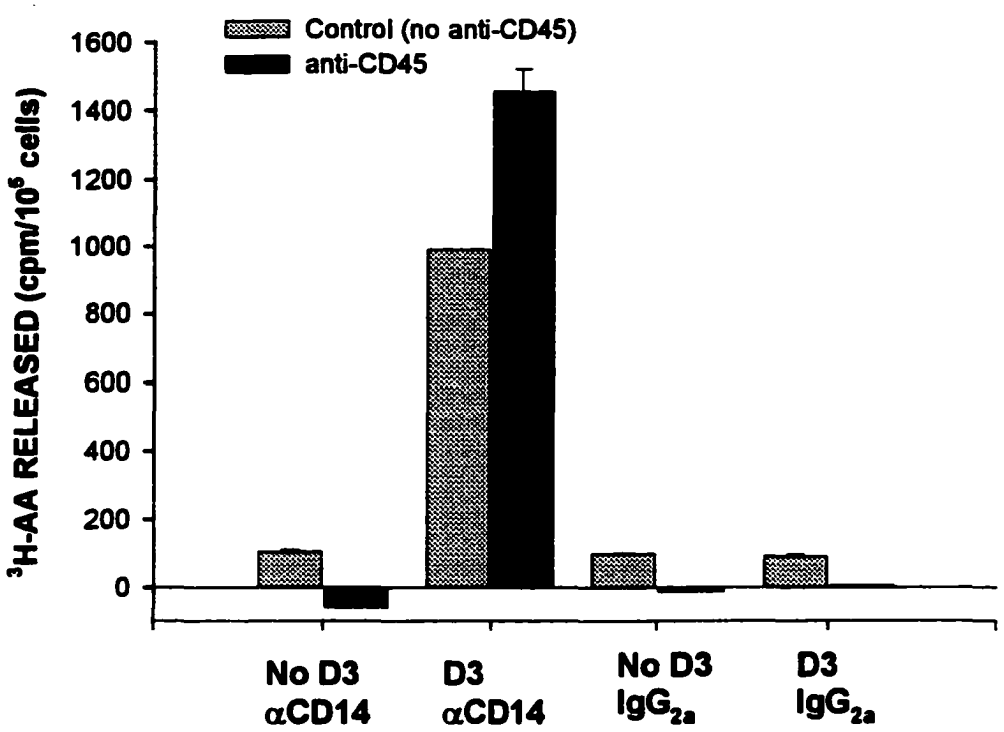


Figure 18. Vitamin D3 pretreatment increased the sensitivity of THP-1 cells to challenge with αCD14, and the effect of αCD45. THP-1 cells, D3 treated or untreated, were labeled and challenged with αCD14 UCHM-1, with or without αCD45 2D1, for 2 hr. Values represent cpm released in 200 μl of cell-free supernatants above control, and are averages of duplicates ± range.

LPS treatment caused increased phosphorylation of CD45. The phosphorylation of tyrosines on CD45 has been shown to modify PTPase activity. The terminal PTPase domain on CD45 appears to be an autodephosphorylation activity (102). In order to determine whether LPS or anti-CD45 mAb affected PTPase activity, the phosphorylation of CD45 was compared under various conditions using anti-

phosphotyrosine mAbs to stain immunoblots of CD45 immunoprecipitates from lysed THP-1 cells. There was an apparent transient increase in the phosphotyrosine stain after 5 minutes of incubation with LPS (Fig. 19).

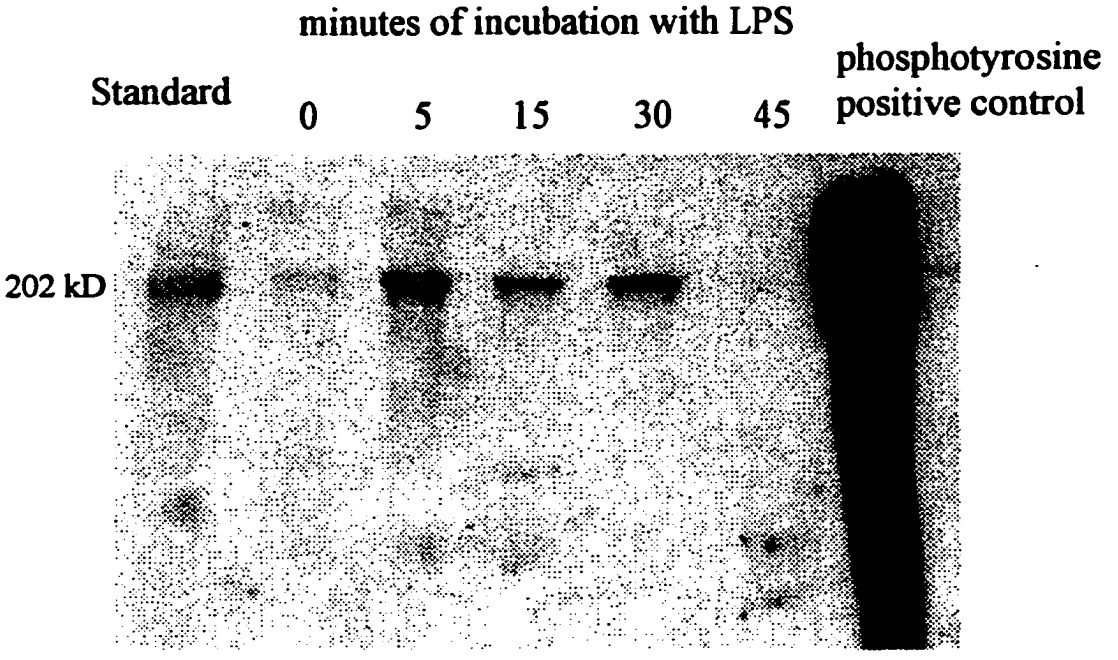


Figure 19. LPS increased phosphorylation of CD45 within 15 min after treatment. D3-pretreated THP-1 cells were treated with LPS for 0, 5, 15, 30, and 45 min (lanes 2-6), and then lysed in lysis buffer containing $NaVO_4$ and PhAsO to inhibit further PTPase activity. Lysates were immunoprecipitated with αCD45 as described in Materials & Methods, and run on 7.5% SDS-PAGE mini-gels. These were then blotted onto PDVF membranes and stained with biotinylated αphosphotyrosine mAb. The immunoblots were developed and films exposed using ECL. Lane 1, Protein standard; Lane 7, positive control.

DISCUSSION

The data reported provide indirect evidence for a role of the CD45 protein tyrosine phosphatase in signaling activated by LPS through CD14. More direct evidence could be obtained by using CD45 knock-out mutants. Our inquiries regarding CD45 knock-out mice or monocyte cell lines were regrettably unsuccessful. Despite the limitations of indirect studies, however, this is the first study to show any relationship between CD45 and signaling for arachidonic acid release, or specifically via CD14. Studies on T cells have now been done that provide both indirect (using mAbs) and direct (using CD45 knock-outs) evidence of CD45 involvement in regulation of signaling (reviewed in (93)). Tyrosine kinases of the src family in CD45-deficient T cells are hyperphosphorylated on tyrosines at inhibitory sites. In these cases, CD45 activates the kinases by removing the phosphate groups from these sites. Because this occurs in resting cells, CD45 apparently serves to maintain a state of readiness prior to TCR-antigen binding. This also implies that CD45 must be in close proximity to these substrates even in resting cells. This is consistent with our data showing constitutively high expression of CD45 on resting cells. Some tyrosine kinases are inactivated by removal of phosphates from positive regulatory sites, so that CD45 can also be a down-regulator of tyrosine kinase activity.

Consistent with this model in T cells, tyrosine kinases appear to be the substrates for dephosphorylation by CD45 in monocytes, and this activity regulates signal transduction (61,77). Signaling through CD14 activates tyrosine phosphorylation (60),

and tyrosine phosphorylation activated by LPS is associated with release of arachidonic acid metabolites (33). This suggests the possibility that signaling by LPS through CD14 activates arachidonic acid metabolism via a tyrosine kinase signaling cascade. This study was undertaken to determine whether CD45 protein tyrosine phosphatase plays a role in regulating that pathway. As suggested in the T cell model, CD45 may simply maintain a state of readiness by keeping tyrosine kinases from being phosphorylated at inhibitory sites. This is supported by the fact that addition of anti-CD45 18 hours before challenge had a greater effect than addition just prior to challenge (Fig. 4a,b), that anti-CD45 had little effect by itself (Fig. 5), and by the fact that anti-CD45 had no effect on desensitization by LPS (Fig. 9). Cross-linking of anti-CD45 appears to have inhibited the phosphatase activity, so that tyrosine kinase signaling was inhibited by phosphorylation (Fig. 8).

The possibility that some of the effects seen were due to binding of anti-CD45 to Fc receptors was considered. However, isotype matched control mAbs had no effect (Fig. 5). Also, activation by anti-CD45 and LPS led to production of PGE_2, but not LTB_4 or LTC_4, consistent with other studies showing that LPS only activates cyclo-oxygenase and not 5'-lipoxygenase (31,33,105), and suggests that anti-CD45 simply increased signaling by LPS, but did not activate other pathways. Activation through $Fc\gamma R$ has been shown to lead to production of LTB_4 from neutrophils and monocytes (49,83).

Several lines of evidence were used to link these effects of anti-CD45 with signaling through CD14. First, it was shown that there was little effect with no serum in the challenge medium (Fig. 11). Signaling via CD14 by LPS requires a serum LPS binding

protein, LBP, for transfer of monomers to CD14 (70,108). Absence of LBP reduces cellular sensitivity to LPS, but LPS can still signal via CD14-independent pathways (68). The reduced effect of anti-CD45 in medium without serum suggests that CD45 may be more active in regulating CD14-dependent signaling than CD14-independent signaling. Similarly, pretreatment of the cells with vitamin D3, which increases expression of CD14 (see Chapter 2) also increased the effect of anti-CD45 (Fig. 12). Without D3 pretreatment, anti-CD45 had almost no effect except at the highest LPS concentration.

Finally, other CD14 ligands, including MPL, DPL, LTA, and an LPS agonist mAb to CD14, also were much more potent for AA release in the presence of anti-CD45 (Fig. 13). However, none of the activating agents that do not bind CD14 showed this synergistic effect with anti-CD45 (Fig. 14.)

There is evidence in T cells that engagement of the extracellular domain of CD45 by other glycosylated cell surface molecules such as adhesion molecules (or CD45-specific mAbs) can modify the protein tyrosine phosphatase activity of the cytosolic domain (93). We attempted to visualize changes in the phosphorylation state of CD45 before and after treatment with LPS, since autodephosphorylation is an indication of PTPase activity (102). This would provide a more direct connection between LPS signaling and CD45. On my initial immunoblots, there was an apparent increase in phosphorylation of CD45 at 5 min after stimulation of the cells with LPS (Fig. 19). These experiments need to be repeated and carefully standardized for the amount of protein/well, and then analyzed by densitometry. This assay could then be expanded to look at the kinetics of autodephosphorylation of CD45, and the effects of this change in activity on function in terms of activation and desensitization of the arachidonic acid metabolism pathway.

Tyrosine phosphorylation has been correlated with all indices of macrophage activation studied so far (24,32-34,37,53,99). Although CD45 appears to play a role in LPS signaling, the broad range of pathways affected by CD45 in macrophages (39,53,61,64,77,101) suggests the possibility that CD45 does not specifically associate with any particular receptor. It may instead affect generalized signaling via src family protein tyrosine kinases (PTK) by maintaining the phosphorylation-dephosphorylation equilibrium at a level that ensures readiness to respond through any pathways utilizing a PTK cascade. If this is the case, it may be possible to reduce sensitivity of macrophages to multiple signals by inactivating CD45. CD45-deficient systems could be used to study this. However, our cross-linking studies suggested that cross-linking CD45 molecules may inhibit the PTPase activity. If cross-linking CD45 during a desensitization period could block response to subsequent challenge with LPS, this would also support the readiness hypothesis. This may therefore be critical to understanding the desensitization phenomenon. It could also provide a mechanism for down-regulation of macrophage activation as a potential therapeutic model. It is along these lines that future studies are being designed.

It was also interesting that in experiments using conditions that favored CD14-independent signaling, there was a consistent slight to moderate reduction in AA release, rather than the increase seen with CD14 ligands. Further studies would need to be done to look at the statistical significance of these reductions. If they were significant reductions, it would be interesting to look at the possibility that CD45 regulates these signaling pathways as well, but in these cases it decreases responsiveness. At least two models are conceivable. First, the kinases involved in CD14 independent pathways may

become phosphorylated on activation sites rather than inhibitory sites, so that CD45 PTPase activity inactivates them. This leads to a conceptual model of a single regulatory system (CD45 dephosphorylation of kinases) that serves to activate some pathways while at the same time inactivating others. This would ensure patterns of responses depending on the type of stimuli present. Another possibility is a kinetic model in which early signaling pathways are maintained in a state of readiness by the constitutive activity of CD45, but binding of a ligand to a receptor in proximity to the extracellular domain of CD45 may alter that activity. Activation of later signaling pathways might then be inhibited due to this change in PTPase activity which may no longer maintain the state of readiness to respond. These models could be explored using a phosphatase assay to determine activity of the CD45 PTPase before and after challenge. We are developing a colorimetric assay for looking at PTPase activity on immunoprecipitates of CD45.

CHAPTER 6

CONCLUSIONS AND FUTURE STUDIES

Elucidation of signal transduction pathways in macrophages will not only improve our overall understanding of these complex and critical players in immune responses, but also may expose exploitable mechanisms for treatment or prevention of conditions caused by immune dysfunction. These studies were undertaken to explore three steps of signal transduction as they relate to LPS activation of macrophages:

1. Recognition of LPS by cells, including factors affecting cellular sensitivity,
2. Signaling components in the activation of arachidonic acid by LPS, and
3. Regulatory mechanisms modulating arachidonic acid metabolism.

It was first necessary to characterize a monocyte/macrophage cell system in order to maximize consistency and reproducibility of results, and to rule out effects of the treatments of other parameters of cellular responses such as viability, receptor expression, and lipid pool labeling. The studies reported in Chapter 2 demonstrate that vitamin D3-pretreated THP-1 monocytic cells provided an excellent cell system for studies on arachidonic acid metabolism.

Macrophages recognize LPS in at least two ways. The primary signaling receptor for LPS is CD14, a glycosylphosphatidylinositol-linked protein that has been shown to be involved in the activation of protein tyrosine kinases and several signaling pathways. LPS also activates cells in a CD14-independent manner, either by some other receptor or by non-specific interaction with the membrane. It has also been proposed that cells

recognize LPS as a mimic of an important intracellular second messenger, ceramide. The work presented in Chapter 3 explored relative potencies of several bacterial amphiphiles, most of which have also been shown to bind CD14, and yet can have very different effects. These data, along with current literature, were used to develop a model of sensitivity of macrophages to LPS based on supramolecular structure and aggregate stability. Binding to CD14 requires presentation as monomers carried by LPS binding proteins. Inaccessibility of monomers to LBP due to supramolecular structure would limit signaling through this pathway. However, aggregate structure might affect CD14-independent signaling very differently than CD14-dependent signaling, so that various amphiphiles might activate the two pathways with very different frequencies, resulting in varying potencies and potentially unique overall responses. This could explain why some LPS preparations are quite toxic while others can be protective *in vivo*. This work in on-going through a collaboration with Ribi ImmunoChem Research.

Chapter 4 explored the LPS/ceramide mimicry model in terms of arachidonic acid metabolism. This model provides a basis for studying not only the recognition of cells by LPS, but also of several signaling components involved in macrophage activation by LPS. The results presented in Chapter 4 showed that the mimicry of ceramide by LPS is not simple, and although they may share some signaling components, LPS has unique functions including the ability to desensitize cells to subsequent challenge and the activation of cyclo-oxygenase. There is a possibility that these two functions are related due to an autocrine effect by prostaglandin or another product related to the cyclo-oxygenase activation. Future studies will be looking at other lipids released by activated

macrophages, and through the use of creative co-culture experiments it will be possible to further investigate the possible autocrine/paracrine effects by released mediators. These studies will be especially important in view of recent evidence that massive endothelial apoptosis in the microvasculature of multiple organs plays an important role in the pathogenesis of septic shock. Ceramide has been shown to be an inducer of apoptosis, and it is released in cells activated by TNFα. This raises the possibility that LPS may not only activate the release of TNFα, but may also further exacerbate the apoptotic events through mimicry of ceramide.

The desensitization phenomenon is intriguing, and the mechanism remains unknown. As mentioned above, there may be an autocrine factor involved. However, even that would not explain what exactly occurs inside the cell to create the refractory state prohibiting further activation of the cell by LPS. Activation and desensitization have both been associated with the activity of protein tyrosine kinases and could therefore be functions of the phosphorylation-dephosphorylation dynamics of these enzymes. Many protein tyrosine kinases are regulated by their own phosphorylation state. In T cells, CD45 deficient mutants are hyperphosphorylated on the src family PTKs, and are unable to respond to antigen binding by the TCR. This provides a model in which hyperphosphorylation causes a refractory, or desensitized state. In order to explore a similar mechanism for desensitization in macrophages, it was hypothesized that CD45 might also maintain a state of readiness to respond by dephosphorylating the src PTKs involved in signaling via CD14. Using anti-CD45 mAbs, it was demonstrated that CD45 does play a role in the activation of arachidonic acid metabolism by LPS. The lack of a

CD45-deficient macrophage cell system or an anti-CD45 mAb that blocked the PTPase activity prevented a careful exploration of the role of CD45 in the desensitization phenomenon. However, cross-linking of CD45 appeared to inhibit the PTPase, and preliminary studies showed that this also desensitized cells to subsequent challenge. This would suggest that blocking CD45 activity prevented the state of readiness to respond by allowing tyrosine phosphorylation on inhibitory sites of the PTKs. However, several more studies would need to be done to determine whether this is also part of mechanism of LPS desensitization.

In summary, three mechanisms of modulating responsiveness to LPS have been examined, including modification of the supramolecular structure of LPS preparations, an autocrine or paracrine effect related to activation of the cyclo-oxygenase pathway, and alteration of the phosphorylation state of PTK signaling cascades. Exploitation of these mechanisms may be possible, and may prove to be therapeutic or prophylactic for pathological conditions resulting from excessive inflammation or other immune dysfunction. Applications may include treatment of asthma, septic shock, cardiac ischemia/reperfusion injury, organ transplants, and atherosclerosis.

The outcome of any set of conditions in a cell or tissue appears to depend not on a linear progression of events, but on the relative frequency of many related events. For example, whether a signal results in mitogenesis or cell cycle arrest appears to depend on the intracellular ratio of two important second messengers with opposing effects: diacylglycerol and ceramide. Also, the activation of tyrosine kinases appears to be due to the relative amount of phosphorylation of the entire cascade, not a simple on/off switch.

The ratio of prostaglandins and leukotrienes appears to be critical in the overall inflammatory response in a given situation, rather than simply the presence of one or the other. Apoptosis is being exposed as a very important part of many processes including beneficial ones (selection of an appropriate set of T cells that survive development in the thymus, destruction of cancerous cells, and embryonic development), and harmful ones (destruction of tissue in septic shock, heart attack, etc). It is known that TNFα is an important inducer of apoptosis, but the identity of the molecule that balances that effect is unknown. It seems very possible that it might be revealed by these continuing studies on the modulation of macrophage activation.

The findings presented in this thesis provide clues to the complexities of LPS-induced signal transduction in macrophages, but the picture is far from complete. The systems of checks and balances for maintaining an effective and appropriate response to bacterial infection without causing undue damage are intertwined in a dynamic network. This prohibits simple answers, and consistently provides more questions with every step forward. In the 1960's, Ivan L. Bennett, Jr., working at Johns Hopkins, expressed both the fascination and frustration researchers face in these complex systems:

"Endotoxins possess an intrinsic fascination that is nothing less than fabulous. They seem to have been endowed by nature with virtues and vices in the exact and glamorous proportions needed to render them irresistible to any investigator who comes to know them." (78).

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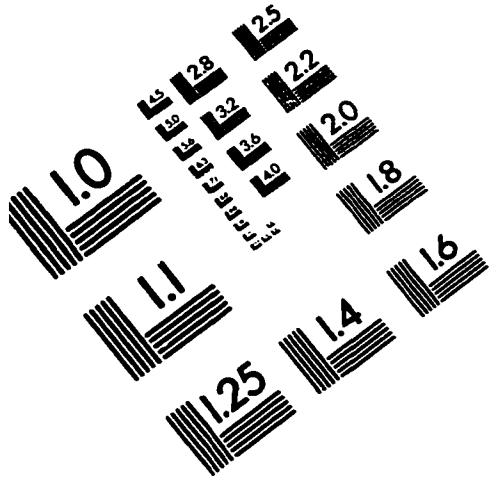
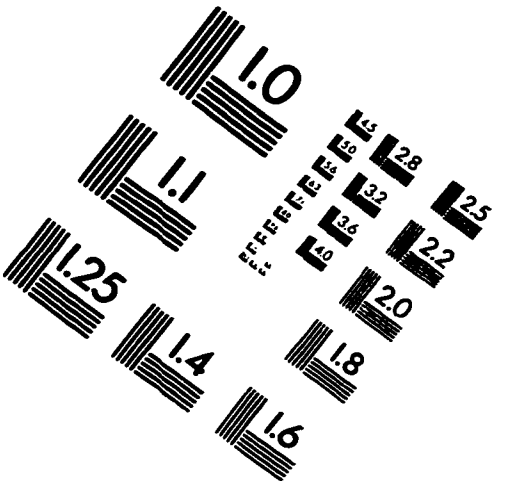
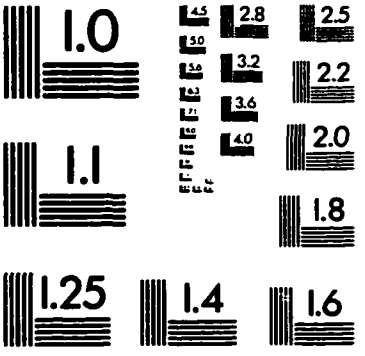
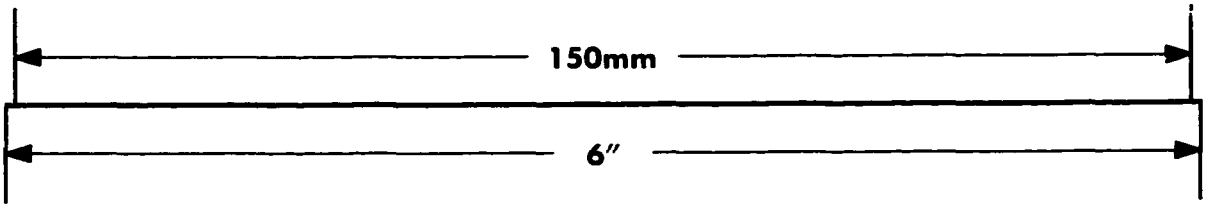
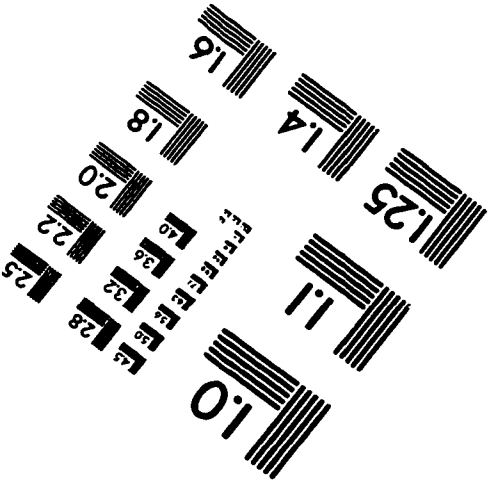
IMAGE EVALUATION TEST TARGET (QA-3)

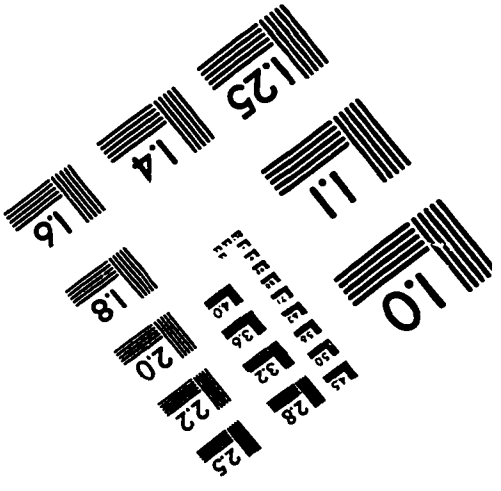












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